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(54) Title: MEANS FOR DETECTING AND PREVENTING HIV INFECTION INVOLVING USE OF RECEPTORS OR BINDING SITES CAPABLE OF INTERACTING WITH GP120

(57) Abstract

The new gp120 receptors or binding sites of the invention are found on CD4+ cells, but differ from the CD4 binding receptor which is blocked when reacting CD4+ cell with a monoclonal antibody directed against the gp120 binding site of CD4, they give interactions with gp120, such as those obtained when reacting gp120 with CD4+ cells in the absence of serum and at a gp120 to cell ratio of 0.5 μg/ml for about 10^6 cells/ml.

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MEANS FOR DETECTING AND PREVENTING HIV INFECTION INVOLVING USE OF RECEPTORS OR BINDING SITES CAPABLE OF INTERACTING WITH gp120

The invention relates to means for detecting and preventing from an infection by the human immunodeficiency virus, HIV, AID causative agent.

It more particularly relates to new receptors or binding sites capable of interacting with gp120 and participating in virus infection. The invention also relates to the biological applications of said receptors or binding sites.

Since 1984, the CD4 molecule was described to be the HIV-1 receptor by which infection occurs (1) and (2) (numbers correspond to the bibliographic references which are given at the end of the description). The HIV envelope protein gp120 binds with high affinity to the CDR2 domain of CD4 (3) and to purified recombinant CD4 (4) and (5). CD4-negative cells could also be infected, which led to the description of other receptors, such as complement receptors (6) and glycolipids in glia cells (7), receptors on fetal astrocytes (8) or on brain capillary endothelial cells (9). A secondary receptor differing from CD4 has also been described on CD4+ Langerhans cells in the skin (10).

By carrying out investigations on the effect of serum on the binding of recombinant gp120 to CD4+ whole cells, in ELISA and FACS, the inventors have identified new binding sites. Such results are in contrast to those on the interaction of rgp120 with isolated recombinant, soluble CD4 reported in (11) and (12).

The inventors have also found accessory molecules for virus in erythrocyte ghosts and have then

explored gp120 binding to erythrocytes and its membrane proteins.

It is then an object of the invention to provide new gp120 receptors or binding sites.

It is also an object of the invention to provide binding sites to said new receptors on the gp120 molecule.

It is another object of the invention to provide serum factors capable to compete with gp120 for the binding to binding sites on CD4+ cells.

The new gp120 receptors or binding sites of the invention are characterized by the fact

- they are found on CD4+ cells, but differ from the CD4 binding receptor since they are involved when gp120 binds to CD4+ cells which are blocked by a monoclonal antibody directed against the gp120 binding site of CD4,

- they give interactions with gp120 such as those obtained when reacting gp120 with CD4+ cells in the absence of serum.

As demonstrated by the examples hereinafter given, the binding of gp120 to CD4+ cells is indeed cited to the so-called CD4 receptor, but involves other interactions which can be identified in the absence of serum and at certain gp120/cell ratios.

Said interactions are considerably inhibited in the presence of serum.

The new gp120 receptors or binding sites of the invention are such as involved in a process comprising incubation of gp120 with CD4+ cells under duration and temperature conditions allowing binding of gp120 to cells with a CD4 blocked receptor.

In such a process, the CD4+ cells are for example pre-incubated for about 1 hour at about 37°C with

a monoclonal antibody specific for the gp120 binding domain of CD4 and gp120 is pre-incubated with bovine serum albumin at about physiological concentration.

The CD4+ cells have intact CD4 as shown by the binding of a labelled monoclonal antibody directed against the gp120 binding site of CD4. At least 90 % of this binding is inhibited by pre-incubation with gp120.

The CD4+ cells are living or dried cells. Such cells are for example, peripheral blood monocyte cells (PBMC) and living or microtiter plate adherent, dried, CD4+ CEM cells.

The gp120 secondary receptors or binding sites of the invention are further characterized by the fact that, in the presence of about 10 % of serum, their interactions with gp120 are inhibited by about 80 %.

More particularly, said secondary receptors or binding sites are capable of interacting in the presence of about 10 % of serum, with 25 to 30 % of gp120, for gp120 concentration of 0.5 and 1 µg/ml and with 40 % of gp120 for 2 µg/ml depending on the gp120/cell ratio.

The new gp120 receptors or binding sites of the invention are still further characterized by the fact that, in the absence of serum, they are capable of interacting with more than 20% of gp120 as shown by FACS analysis.

More particularly, said new receptors or binding sites are capable of interacting with about 80% - 90 % of gp120 at gp120 doses of 0,5 or 1 µg/ml, and 50 % - 60 % at 2 µg/ml, in ELISA with BSA at about physiological concentration and in the absence of fetal calf serum or human serum.

Such results demonstrate the secondary interactions of gp120 with whole living cells.

The interaction is more pronounced on dried CEM cells with accessible intracellular receptors.

Said new gp120 receptors or binding sites are localized on the outer cell membrane of living cells. They appear to possess lower affinities than the CD4 receptor, since they appear to be present on all cells, as shown by double labelling, but bind about at least 20% of total bound gp120.

Binding to said receptors is considerably increased when intracellular sites become available, as found with dried cells by ELISA or with different kinds of permeabilized cells by FACS analysis.

The new gp 120 receptors or binding sites are further characterized by the fact that their binding activity for gp 120 are not destroyed when CD4 is deleted on CD4+ cells, for example by trypsin, formaldehyde or deoxycholate.

Accordingly, they are more resistant than said CD4 receptor to proteolytic enzymes and chemical alteration.

Said gp120 receptors or binding sites will also be hereinafter designated as serum inhibited receptors (SIR).

Investigation experiments carried out by the inventors have shown a strong interaction between gp120 and the proteins belonging to the families of the multi-passage transmembrane proteins, such as present at the surface of human erythrocytes, or lymphocytar cells such as CEM cells.

Accordingly, the invention relates to new gp120 receptors or binding sites such as above defined, further characterized by the fact they comprise at least a part

of the proteins of multi-passage transmembrane protein families, or lymphocyte cells such as CEM cells.

Transmembrane proteins comprise proteins whose MW is in a range of about 40 kDa to 150 kDa.

For example, multi-passage transmembrane proteins in erythrocyte comprise band 3 protein (100 kD), glucose transporter (54 kD), Rh50 (44 kD), calcium transporting ATPase (134 kD), sodium transporting ATPase (112 kD).

In order to examplify the invention, it will hereinafter be referred to proteins of band 3-like protein family, although the invention encompasses any multi-passage transmembrane protein capable of interacting with gp120 under the conditions disclosed in the examples.

Said band 3-like proteins are known to be ubiquitous and may be found in the plasmic membrane, the Golgi apparatus, as well as in mitochondria in various cells.

It comprises proteins having homology, i.e. the so-called band 3 protein which, when originating from erythrocytes, possesses a MW of 102 kD, band 3 AE 2 (AE = anion exchange) with a MW of 112 kD, and band 3 AE3 having a MW of 137 kD (the MW were evaluated in SDS-PAGE).

According to the cDNA deduced amino acid sequence (13) band 3 protein from erythrocytes (EXPASY, Swiss-prot P 02 730) possesses 911 amino acids, several hydrophobic regions allowing to predict 12-14 transmembrane passages, a 40 kD C-terminal and a short N-terminal cytoplasmic tail (14). The 4 major extracellular loops contain approximately 14 positively and 6

negatively charged amino acids with a ratio of 2.3 in favour of the positive charges. The domination of the positive charges could have a physiological role for anion exchange as they seem to constitute a positively charged funnel contributing to repulsion of cations and selection for anions (15). Band 3 AE 2 protein with a total of 1240 AA and a 303 AA larger cytoplasmic tail (EXPASY, Swiss-protP 04 920) is an anion exchanger of wide distribution, first described for the leucocyte derived cell line K562 (16). Band 3 protein AE 3 is very similar with 1232 AA containing also a 303 larger cytoplasmic tail (EXPASY, Swiss-prot P 48 751) and is localized in heart and brain. Band 3-like protein A2 present in lymphocyte derived cells (16 EXPASY Swiss-prot.) were shown to have high crossreactivity with rabbit antibodies to erythrocyte band 3 protein.

According to the invention, the receptors or binding sites of HIV gp120 are thus characterized in that they comprise at least a part of proteins of the band 3-like protein family.

The experiments carried out by the inventors, which are given hereinafter in the examples, have shown absorption of gp120 by band 3 from erythrocytes or competition of band 3 protein with CD4 blocked CEM cells for gp120 binding or virus infection. The high crossreactivity between both band 3-like molecules indicates the involvment of this kind of transmembrane molecules as accessory receptors for virus infection.

The invention particularly relates to the gp120 receptors or binding sites comprising at least a part of band 3 protein such as present on human erythrocytes, or

lymphocyte cells such as CEM cells, particularly on the outer membrane surface.

Said receptors or binding sites are also characterized in that they are capable of interacting with gp120, in the absence of human serum, at 37°C.

The interaction between gp120 and band 3 protein is indeed inhibited by serum.

Said receptors are such as present on HIV-infectable cells, such as CD4+ CEM cells.

The invention also relates to transmembrane proteins, with several passages from inside to outside of the cell, under a purified form, and the fragments thereof, particularly the extra-membrane regions, especially those positively charged, inasmuch as said fragments and regions have a specific binding activity with respect to the gp120 present on HIV.

The term "protein", such as used in the description and the claims, designates a protein of the transmembrane protein families, under a purified form, or in vesicles or in reconstituted liposomes, as well as the fragments or regions of the protein, such as above identified.

Said term also encompasses the recombinant forms of the protein, for example the fusion proteins, or the forms which are totally or partially devoid of their intramembane sequence.

The invention relates to the purified band 3 like proteins separated from at least partially of the membrane associated proteins. Particularly, such purified proteins, especially band 3 proteins, are practically totally devoid of spectrin, ankyrin, only once crossing

transmembrane proteins such as glycophorin and complement receptor CR1.

As already above-mentioned, it is specifically referred to band 3 protein to examplify the invention, but said expression designates any other multi-passage transmembrane protein and correspond to the accessible transmembrane proteins on the outer membrane surface, or fragment thereof, inasmuch as they are able to react with gp120 as above defined.

According to another aspect, said new gp120 receptors or binding sites are such as involved when reacting gp120 with erythrocytes adsorbed to plates or pretreated by high proteolytic enzyme concentrations of potent enzymes, such as Pronase ^{R}.

The invention also relates to the polyclonal or monoclonal antibodies directed against said protein. The fragments of said antibodies, particularly the Fab region are also included in the invention.

The invention also relates to anti-Ig antibodies, i.e. the anti-idiotype antibodies.

Said anti-idiotype antibodies are capable of reacting, according an antigen-antibody reaction, with the above defined antibodies. The invention also covers the fragments of said antibodies.

The polyclonal antibodies are obtained according to usual methods by immunizing animals with band 3 protein, or fragments or regions thereof, recovering the antibodies obtained from splenic cells, and purifying the same.

Monoclonal antibodies are obtained by cultivating hybridoma, under the usual conditions which comprise : fusioning splenic cells secreting antibodies

with myeloma cells, selecting those clones secreting hybridomas, and injecting said clones to mice for producing tumoral ascites from which the desired monoclonal antibodies are recovered and purified.

The above defined proteins and antibodies are useful for elaborating detecting means and therapeutic or prophylactic compositions.

The invention thus relates to detection or diagnostic means, compositions and kits, comprising said proteins or said antibodies in a sufficient amount to characterize an HIV infection and, optionally, to quantify the HIV level in a sample to be analyzed or the immune response against HIV.

In detection or diagnostic compositions, the proteins or the antibodies advantageously comprise a marker revealing the immunological reaction.

According to the invention, the method for detecting the presence of HIV or the immune response comprises :

- contacting the sample to be analyzed with the secondary receptor such as above defined, said receptor being capable to bind to the HIV-RIS reaction product optionally contained by the sample, or to the SIR site, or alternatively, with the above defined protein, capable to bind to HIV, where the inhibition of the fixation of the viral proteins on SIR will indicate the presence of antibodies capable to hinder the virus fixation on said receptor,

- revealing the antigen-antibody reaction when occurred.

The contacting step is carried out under appropriate conditions, particularly with respect to the

duration of the reaction, the temperature, and the buffer, so as obtain the HIV fixation when HIV is present.

The sample to be analyzed is more particularly a body fluid such as blood, plasma, urine, salive, cerebro-spinal fluid, seminal liquid.

Antibodies or proteins are used in solution or fixed on a support.

Labelling means are used to reveal the reaction, such as fluorescent agents for example fluorescein, enzymes like peroxidase, or coloring agents.

The detection of the presence of HIV or anti-HIV antibodies in view of studies or diagnostic, will be advantageously carried out by using a kit comprising said protein or said antibody, optionally fixed on a microplate, in a sufficient amount for performing the test, reagents and buffers for the detection as well as instructions for use.

According to another aspect, the invention relates to compositions useful for therapy, comprising, in association with pharmaceutically acceptable carriers, an efficient amount of at least one antibody, or one protein, such as above-defined.

The antibodies are thus used as inhibitor agents with respect to the interaction with HIV.

Band 3 protein, or its fragments, is used to compete with the transmembranar protein the SIR site cells.

Advantageously, the pharmaceutical compositions of the invention comprise agents capable of protecting the antibodies or proteins administered to a patient against the effect of cellular proteases.

The composition of the invention may also be used in association with antiviral agents.

For such therapeutical applications, the proteins or the antibodies are administered under galenic forms for parenteral or intravenous administration.

They are also administrable as liposomes.

Gp 120 used in the investigation experiments is a recombinant glycosylated gp120 from HIV-1 from baculovirus. But the intact virus with oligomeric gp120 may be used instead of the recombinant gp120 (rgp120). Inhibition of virus infection in vitro by band 3 protein vesicles, as shown by the examples hereinafter given, demonstrates indeed that virus also possesses very similar structures to recombinant gp120 concerning the interactions with said new receptors or binding sites.

The term "gp120" as used in the specification and the claims encompasses all the forms of gp120, i.e. the recombinant form or the natural viral form. The invention also relates to the part of gp120 such as bound on living cells, distinct from the binding to CDR2 of CD4, when incubating CD4 + cells with monoclonal antibodies specific for the gp120 binding domain of CD4.

The presence of gp120 on the cells is revealed with an anti-gp120 rabbit anti-serum followed by an anti-rabbit Ig coupled to peroxidase for OPD coloured reactiver as usually done in ELISA. The corresponding binding site on the gp120 molecule is characterized by the fact that it is localized, with monoclonal antibodies, near the V3 and the CD4 binding region. The localization of the binding region near the V3 region is

highly interesting, since it is known to interact with neutralizing antibodies.

Taking into account the low efficacy of CD4 blockade observed on dried cells in ELISA and in FACS analysis, it is believed that the gp120 molecule possesses at least two binding sites, with different specificities, one for the CD4 receptor and an other for said new receptors or binding sites. Gp120 might then bind to both cellular sites simultaneously, thereby inducing the formation of a bridge between both cellular receptors.

The invention also relates to the part of gp120 whose binding to said outer cell membrane sites of living cells can be partially inhibited by pre-incubation of gp120 with fetal calf serum or human serum.

It will be appreciated that said gp binding site is of great interest for determining the amino acid and the corresponding nucleotidic sequences involved in the binding to the new receptors and for studying their anti-HIV effect.

Polyclonal and monoclonal antibodies directed against the binding sites situated on the gp120 molecule are also part of the invention as well as their diagnostic on therapeutic applications.

The invention also relates to the serum factors capable of at least partially inhibiting gp120 binding to said new gp120 receptors or binding sites.

Said serum factors are then characterized by the fact they compete with gp120 binding on said gp120 receptors or binding sites and/or modify the gp120

molecule itself by direct binding or by partial degradation.

The serum factors and the fractions are useful to study the inhibitory effect of the VIH infection of target cells. The results obtained concerning said gp120 receptors or binding sites give useful tools to test the validity of vaccinating substances.

The invention will be further illustrated by the following examples taken in conjunction with the accompanying drawings, wherein

- Figures 1A and 1B represent the binding of gp120 or of mAB F101.69 to CEM cells and inhibition of mAB binding by gp120 (FACS),
- Figures 2A and 2B, the gp120 binding on CD4 + CEM cells in presence of blocking mAB F101.69 or of 10% FCS or both (FACS),
- Figures 3A to 3C, and 4A to 4C, the gp120 binding to treated or dried cells under different conditions (FACS and ELISA respectively),
- Figure 5A to 5D, the double labelling of blocked CD4 receptor and cell bound rgp120 (FACS),
- Figure 6, the double labelling of gp120 and blocked CD4 on CEM cells in the cytofluorometric microscope.
- Figure 7, the localisation on the gp120 molecule of the binding site to the new receptors,
- figures 8A and 8B, the total binding to dried CEM cells or specific binding to CD4 after prior iodination of gp120 by idogen,
- figures 9A and 9B, the inhibition of gp120 binding by mAB F101.69 or serum, or both together,
- figures 10A and 10B, the gp120 binding to human erythrocytes and the inhibition thereof by serum,

- figure 11, the SDS electrophoresis of highly enriched band 3 protein,

- figure 12, the gp120 binding to purified transmembrane proteins from erythrocytes,

- figure 13A and 13B, the inhibition of gp120 binding to CD4 + CEM cells by human serum and band 3 protein in erythrocyte vesicles, respectively,

- figure 14, the absorption of gp120 by enzyme treated erythrocytes or band 3 protein vesicles, and

- figure 15, the inhibition of RT after adsorption of HIV to band 3 vesicles.

MATERIAL AND METHODS

MATERIALS

Microtiter plates were purchased from NUNC (Roskilde, Denmark) ; bovine serum albumin (BSA) ; glycophorin, octyl-glycopyranoside from Sigma (St. Louis, M.O.) ; Pronase ®, inhibitor and trypsin inhibitor from Boehringer (Mannheim, Germany) ; trypsin from Serobal, FCS from Gibco ; 4-15 PAGE SDS gels from Pharmacia (Upsala) ; biobeads SM-2 from Bio-Rad (Richmond, Ca.) erythrocytes and normal human serum from the Regional Blood Distribution Center (Montpellier)

Antigens and antibodies

Monoclonal mouse antibodies (mABs) known to block gp120 binding to the CD4 receptor, F101.69 and F92.3A11 (Sanofi Research, Montpellier, France) and OKT4a (purchased from Ortho Diagnostics, France) were used. The mAB F101.69 had been classified as an anti-CD4 antibody during the Third International Workshop on Human Leukocyte Differentiation Antigens (14). It binds to the CDR2 domain of CD4, also known to bind gp120 from HIV-1, and is inhibited by mutations of amino acids 42-43 (15).

Recombinant, glycosylated gp120 from HIV-1/IIIB produced in baculovirus was used (ABT, now Intracell purchased from NEOSYSTEM, Strasbourg, France, or AGMED, Bedford, MA, USA).

Gp 120 was labelled by antigen-antibody complex formation with anti-HIV mAB 110.4 (2 molar excess) which was directed against V3 of gp120 (GENETIC SYSTEMS). This antibody had been conjugated before (16 and 17) with periodate (PROLABO, Paris, France) to peroxidase (BOEHRINGER, Mannheim, Germany). The peroxidase labeled antibody in the absence of gp120 served as a specific control for every experimental point and was subtracted from the experimental values. Unlabelled gp120 was revealed with an anti-gp120 hyperimmune antiserum from rabbits, followed by peroxidase conjugated to anti-rabbit Ig from sheep (Amersham, England). The rabbit anti-gp120 antiserum was obtained after a first immunization with gp120 in complete Freund's adjuvant, followed by monthly hyperimmunizations without adjuvants in doses of 1 µg/kg.

The rabbit anti-gp120 antiserum was obtained after a first immunization with gp120 in complete Freunds adjuvant, followed by monthly hyperimmunizations without adjuvants in doses of 1 µg/kg. Anti-rabbit Ig and anti-mouse Ig conjugated to peroxidase came from Amersham, England, and anti-rabbit Ig or anti-mouse Ig conjugated to FITC or phycoerythrin from Sigma, France.

sCD4 was obtained from the NIH AIDS Research and Reference Reagent Program (cat. n° 1813, lot 394150 and cat. n° 1246, lot 1CD51009) and also from ABT, now Intracel, Cambridge, MA (cat. n° 13001, lot 55-90-1A).

Cells

Peripheral blood mononuclear cells (PBMC) were freshly isolated from healthy donors with Ficoll-Paque (PHARMACIA, Paris, France). CD4+CEM cells were obtained

from the ATCC, USA, and cultured in RPMI plus 10% FCS. Microtiter plate adherent dried cells, (18), were obtained after three washes of CEM cells in PBS without addition of proteins and distribution of 10^5 CEM cells/well in non saturated maxisorb U-microtiter plates (50 µl per well). Cells were dried overnight at 37°C in closed boxes with silica gel (Prolabo, Paris, France) distributed around the microtiter plates. Before use, the wells were saturated with 3% BSA. Non-adherent dried cells for FACS analysis were obtained in the same way, but in microtiter plates after prior saturation with BSA 3% to avoid adherence of cells.

ELISA

After saturation of U.maxisorb microtiter plates (Nunc, Roskilde, Denmark) with 3% BSA for 30 min at 37°C (RIA grade, Sigma) 10^5 living cells, human PBMC or CEM (ATCC, USA) were distributed per well and washed twice with PBS+BSA 0.3% (200 µl/well). Gp120 was pre-incubated with 10% FCS (decomplemented 30 min. at 56°C) or 3% BSA for 1 h at 37°C and then incubated in quadruplicates with the cells for 30 min. at 37°C in the indicated incubation buffer (PBS plus BSA 3% or 10% FCS). Cells were then washed twice in PBS with 0.3% BSA and further incubated (30 min. at 37°C) with a 1/1000 dilution of a rabbit anti-gp120 antiserum. After 2 washes cells were incubated with a peroxidase labeled anti-rabbit Ig from sheep (F(ab)'2, Amersham, UK) for 30 min. at 37°C. After another 3 washes peroxidase was revealed with OPD and stopped with H_2SO_4 4N. Optical densities were read at 492 nm in a Multiskan (Flow Laboratories, France).

In blocking experiments, cells were incubated (30 min. at 37°C) with CD4 blocking antibodies F101.69 or OKT4a at 10 µg/ml concentrations in PBS-BSA 3% followed

by two washes before reaction with rgp120. In some experiments (as indicated), the blocking mAB was not washed away before incubation with rgp120.

Every ELISA plate included two internal standards of 0.5 µg/ml gp120 in BSA 3%, which served as references for comparison between different microtiter plates. Experimental values were expressed as ratios of the corresponding reference values. Optical densities of references varied between 1.0 to 2.0. For each experimental quadruplicate, controls consisted of quadruplicates containing peroxidase labeled antibody without gp120, the values of which were subtracted from the experimental values. The control for peroxidase labeled F101.69 consisted of a similarly labeled mAB at the same concentration. Wells without cells, but saturated with BSA 3%, indicated low nonspecific rgp120 binding to the wells, which never exceed 5%.

Inhibition studies of the binding of gp120 to cells were effected with anti gp120 mABs after prior incubation of gp120 with the mABs at 10 µg/ml for 1h at 37°C. The mixture was then incubated and further processed on dried cells, as described.

In experiments with erythrocytes anti-species Ig antibodies linked to alkaline phosphatase were used and revealed with nPPD.

Analysis by a fluorescence activated cell sorter (FACS)

Experiments with CEM cells for FACS analysis were carried out in microtiter plates under the same conditions as for ELISA. Only during the last step was anti-Ig coupled to peroxidase replaced by anti-Ig antibodies linked to fluorochromes at determined optimal dilutions. For dual fluorescence analysis mouse mAB F101.69 directed against CD4 was revealed with anti-mouse

Ig-FITC and rgp120 incubated with rabbit anti-gp120 antiserum with anti-rabbit coupled to phycoerythrin. During FACS analysis (FACSort, Becton Dickinson) homogeneous cell populations ($5x10^3$ cells) were selected according to scatter parameters and then analyzed for mean fluorescence intensity (MFI) for each fluorochrome. An internal reference consisted of 2 µg/ml rgp120, which was run in all experiments. In order to compare different experiments with each other by mean values and standard deviations, the reference MFI was defined as 1 and all experimental values were transformed into relative MFI values by calculating the corresponding ratios (exp./ref.).

In some experiments, cells were incubated with propidium iodide (PI) from Sigma, France for 10 min. at 4°C before FACS analysis, to determine and exclude dead cells. In such experiments rgp120 was revealed with rabbit anti-rgp120 antiserum followed by anti-rabbit Ig-FITC (Sigma).

The Kruskal-Wallis test, a Chi-Square approximation,was used for the statistical analysis.

Cyto-fluorimetric microscopy

CEM cells (10^6 cells per ml) were prepared and stained as for FACS analysis. Cells were then concentrated by centrifugation, layered on a slide and air dried at room temperature. Sections were analyzed on the scanning stage of the ACAS 570 Interactive laser Cytometer (Moridian, Okemos, MI, USA). Dual fluorescence expression was derived from pseudo-color two-dimensional image scans generated by a laser beam and an X-Y scanning stage (0.800 µM) and analyzed with two detectors each generating an image. Pseudo color linear scale range from 0 to 4096, 570 and 930 nM filters were used for PE and TRI-COLOR analysis respectively.

CD4 deletion

Living or microtiter adherent dried cells were after saturation with BSA 3 % incubated in microtiter plates (30 min, 37°C) with increasing concentrations of trypsin, paraformaldehyde or deoxycholate.

After treatment, cells were immediately washed three times in PBS-BSA 0.3 % and resaturated with BSA 3 %.

The presence of CD4 was detected in ELISA by incubation of treated cells with the F101.69 mAB linked to peroxidase (30 min, 22°C) and revealed with OPD. For FACS analysis cells were incubated with F101.69 (30 min, 37°C), followed by an anti-mouse IgG-30 min, 22°C), similar to the above described method. Binding of gp120 after incubation with cells (1h, 37°C) was studied by further incubation with rabbit anti-gp120 antiserum (30 min, 22°C) revealed with an anti rabbit Ig POD (30 min, 22°C) and OPD for ELISA or anti-rabbit Ig (30 min, 22°C) for FACS.

Iodination of gp 120

Iodogen, 70 nmol (30 µg in 1 ml chloroform), was precoated on glass tubes (14 x 100 nm) by evaporation under nitrogen. After one wash in citrate phosphate buffer pH 7.4 and complete elimination of liquid, 50 µg/ml of rgp120 were incubated in a final volume of 180 µl (citrate phosphate buffer pH 7.4 with 0.5 % BSA) containing 0.3 µg/ml cold $NaIO_4$ with the iodogen coated tube under continuous stirring with a vortex. After different time periods the reaction was stopped by transfer of gp120 to an other tube and immediate 1:20 dilution in PBS containing 3 % BSA and 0.2 mg/ml tyrosine.

Modified gp120 was examined for its binding activity to CD4 by inhibition of the mAB F101.69 to bind

to CD4. After saturation of microtiter adherent CEM cells with PBS-BSA 3 % cells were incubated with the modified gp120 (1h, 37°C) and after two washes with PBS-BSA 0.3 % further incubated with F101.69 linked to peroxidase (30 min, 22°C) and revealed with OPD.

Binding of modified gp120 to said gp120 receptor or binding site was tested by its binding to dried CEM cells, which predominantly show binding to said receptor or binding site. After saturation with 3 % BSA cells were incubated with modified gp120 (1h, 37°C) and, after 2 washes, further incubated with an anti-gp120 rabbit antiserum (30 min, 22°C) followed by an anti-rabbit Ig coupled to peroxidase (30 min, 22°C) and revealed with OPD.

Modification of the gp120 structure was tested by adsorption of unmodified or modified gp120 at the concentrations of 25 µg/ml and two 1:4 dilution on maxisorb microtiter plates (1h, 37°C). After saturation with BSA 3 %, wells were incubated with a rabbit anti-gp120 antiserum (30 min, 22°C), followed by anti-rabbit Ig-POD (30 min, 22°C) and OPD. At 50 % inhibition the concentrations of modified and unmodified gp120 were compared to each other.

Enzyme treatment

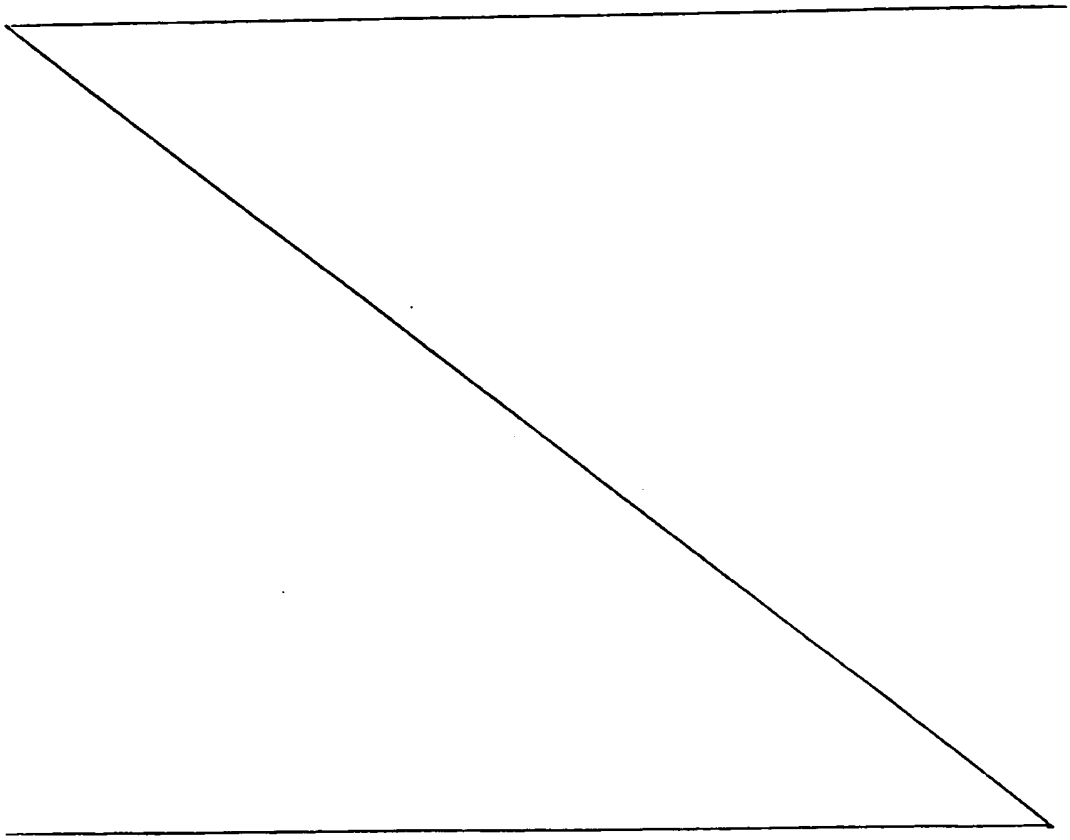
Freshly taken erythrocytes from healthy donors were washed 3x in PBS, incubated with Pronase^{R} , trypsine in PBS for 1h at 37°C under agitation, washed 3x in PBS + BSA 0.3 % + inhibitor and then incubated with 2 µg/ml gp120 for 1h at 37°C under agitation. After centrifugation the supernatant was incubated with microtiter adsorbed CEM cells and gp120 binding revealed by the inhibition.

RESULTS

1- INHIBITION OF GP120 BINDING TO WHOLE CELLS BY CD4 BLOCKADE WITH mABs (ELISA)

Gp120 from HIV-1/IIIB was preincubated with 10%FCS and controls with BSA 3% for 1 h at 37°C, PBMC, living CEM or microtiter plate adherent, dried CEM cells were saturated with 0.3% BSA and incubated with the anti-CD4 mABs: F101.69 or OKT4a and control with BSA or anti-CD2 mAB F92.3A11 for 30 min. at 37°C. Cells were further incubated with the pre-incubated gp120 for 1 h at 37°C in the indicated pre-incubation medium. Gp120 complexed to mAB 110.4 which was linked to peroxidase, could be directly revealed with OPD. Unlabelled gp120 was further incubated with rabbit anti-gp120 antiserum followed by peroxidase labelled sheep anti-rabbit Ig and then revealed with OPD.

The results are given in Table 1 (experiments A to K)



	Incubation medium	control cell medium		% gp120 binding on CD4 blocked cells					means	SD'		p ≤
			OKT4a	F101.69 (anti CD4 mAB)								
				PBMC (living)								
A	FCS 10%	BSA or aCD2		23	23	5	17	28	19	8.8		
B	BSA	BSA		77	83	74	72	79	77	4.4	A vs B	0.01
C	BSA	aCD2	79	82	67	77	86	84	79	7.7	A vs C	0.01
											B vs C	n.s.
				CEM cells (living)								
D	FCS 10% :	BSA or aCD2		3	2	16	17	26	13	9.9		
E	BSA	BSA		77	77	72	85	82	79	5.1	D vs E	0.01
F	BSA	aCD2	88	79	81	98			86	10.3	D vs F	0.01
											E vs F	n.s.
				CEM cells (dried)								
G	FCS 10%	BSA or aCD2		83	76				80	5.0		
H	FCS 100%	BSA or aCD2		25	22				24	2.7		
J	BSA	BSA	90	98	97	98	99	98	98	0.9	(G+H) vs J	0.02
				Different gp120 lot on CEM cells (living)								
K	BSA	BSA		51	45	62	34		48	11.7		

. Compared to anti-CD2 mAB F92.3A11 instead of BSA
. SD : standard deviation
n.s. = not significant

gp120 binding to PBMC in ELISA

In order to favour serum interactions, recombinant gp120 from baculovirus was pre-incubated with 10% FCS (1h, 37°C) and further incubated with PBMC in 10% FCS (30 min. at 37°C). It bound to the cells with optical densities > 1, which were considered as 100% binding. Preincubation of PBMC with mAB F101.69 which recognises the gp120 binding region in CD4 and inhibits gp120 binding, reduced bound gp120 to 19%, as compared to controls (Table 1A, 5 experiments). Therefore gp120 in presence of FCS bound essentially to the CD4 receptor, as expected from the literature.

The influence of serum on gp120 binding was now tested by omitting serum completely. Gp120 was preincubated in BSA 3%, an almost physiological concentration, and further incubated with PBMC also in BSA 3%. Gp120 bound to the cells and its OD was considered as 100% gp120 binding to control cells. Other target cells were first blocked at the CD4 receptor with mAB F101.69 and then incubated with gp120. Now, the blocking antibody reduced gp120 binding only to 77% with respect to controls (Table 1B, 5 experiments). The results in absence and presence of FCS were significantly different (Table 1, A vs. B : $p < 0.01$). This means that, in the absence of FCS, about 80% of cell bound gp120 was not inhibited by the anti-CD4 antibody and appear to be directed to an other cellular binding site, different from the known CD4 epitope.

It was verified that the blocking mAB F101.69 was in sufficient excess to inhibit gp120 binding to CD4 completely. In all experiments, the antibody was used at 10 µg/ml, which is more than 10 x higher than saturation concentrations in ELISA. It was also tested, if the blocking antibody was directed against the binding site

of gp120 on CD4. Preincubation of the cells with 2 µg/ml gp120 inhibited subsequent binding of mAB F101.69 by more than 80%.

Since it is possible that the blocking mAB could have altered the surface characteristics of the target cells, while the control in BSA without mABs were unchanged, complementary experiments were carried out, in which the controls were exposed to mAB F92.3A11, directed to the unrelated CD2 receptor. Such experiments were first effected in the presence of FCS and gp120 binding could be reduced to 20% by mAB F101.69 (table 1A). When the experiments were effected in BSA 3% i.e. without FCS the blocking mAB reduced gp120 binding only to 79% with respect to the contraol cells (Table 1C). The results in absence and presence of FCS were highly significant (A vs. C $p<0.01$). When target cells were blocked with the reference mAB OKTa, instead of mAB F10169, identical results of 79% were obtained (Table 1C). This confirms that the residual gp120 binding should be directed to a receptor different from the classical CD4 receptor, which is only diminished in the absence of serum.

. Gp120 binding to CEM cells in ELISA

PBMCs are a heterogeneous population containing different cell types, i.e. CD4+, CD8+lymphocytes and monocytes. In order to show that secondary gp120 binding can be found on purified CD4+ cells, CEM cells were examined in the same way as PBMCs. When experiments were effected in the presence of 10% FCS, blockade of CEM cells with the anti CD4 mAB F101.69, reduced gp120 binding to 13% (Table 1D), indicating predominant gp120 binding to the CD4 receptor. However, in experiments performed in the absence of FCS, the same blocking antibody reduced gp120 binding only to 79% (Table 1E). The differences were highly significatn (D vs.E: $p<0.1$),

whether control cells were maintained in BSA (79%) or incubated with the control mAB anti CD2 (86%) (Table 1F). Replacement of mAB F101.69 by the reference mAB OKT4a also reduced gp120 binding to 88% only (Table 1F). This confirms the existence of gp120 binding to secondary receptors on CEM cells in the absence of serum. The secondary binding sites and CD4 are simultaneously present on the same CEM cell.

When similar experiments were effected with another gp120 lot, slighty different values were obtained. In the absence of serum mAB F101.69 reduced rgp120 binding on CEM cells to about 50% instead of 80%. Therefore gp120 binding to secondary receptors may be influenced by the gp120 lot used and could be generally estimated by ELISA in the range of 50% - 80% of total bound rgp120 (Table 1K).

. Gp120 binding to dried CEM cells in ELISA

In order to exclude modulation of DC4 during incubation at 37°C as a responsible factor, which could have diminished the total amount of accessible CD4 receptors, microtiter adsorbed dried CEM cells were studied, which take up trypan blue, do not undergo endocytosis but present accessible membrane and intracellular receptors. It was verified with the corresponding mABs that CD4 binding sites for gp120 were fully expressed on dried cells, as were CD5 and CD7, although CD2 had disappeared. When experiments were performed in the presence of 10% FCS, blockade of CD4 reduced gp120 binding to 80% (Table 1G), which is much less than on living cells. An increased FCS concentration of 100% reduced gp120 binding to 24% (Table 1H), showing a dose dependent effect of FCS. When experiments were effected in BSA i.e. in the absence of FCS, there was no significant reduction of gp120 binding (95%) (Table 1J)

and this was also observed with the reference mAB OKT4a (Table 1J). Binding of an other glycoprotein to dried CEM cells, such as peroxidase, was not significantly increased. In these experiments, peroxidase conjugated to mAB 110.4 was used in the absence of gp120 and its binding to dried cells did not exceed 10 - 20%. Therefore secondary binding sites on dried cells are inhibited by FCS in a dose dependent manner, requiring high concentrations for total inhibition. Modulation of CD4 could be excluded as a responsible factor for decreased binding to CD4 receptors.

2- GP120 BINDING TO CEM CELLS BY FACS ANALYSIS

The existence of secondary binding sites was verified by a second method, FACS analysis, on living CEM cells.

Binding of gp120 or of mAB F101.69 to CEM cells and inhibition of mAB binding by gp120 : the results are illustrated on figures 1A and 1B.

A) CEM cells were first incubated with rgp120 (1h, 37°C) and then with anti-gp120 rabbit antiserum, followed by anti-rabbit Ig-phycoerythrin (black squares) or, after incubation with rgp120, CEM cells were incubated with anti CD4 mAB F101.69 which was detected with anti mouse Ig-phycoerythrin (open squares). Results were expressed as relative mean fluorescence intensities (MFI), which are the ratio of experimental versus internal standard arbitrary units.

B) CEM cells or PBMC were incubated with increasing concentration of mAB F101.69 (30 min. 37°C) and then revealed with an anti mouse Ig-FITC by FACS. (mean values of 4 experiments with different donors for PBMC, compared by the intern reference for 10 µg/ml F101.69).

Binding of gp120 to CEM cells was saturable. It increased in a dose dependent manner up to plateau levels at about 30 µg/ml (Fig 1A). There is competition between gp120 and mAB F101.69 on the same binding site, since after incubation with 10 µg/ml of gp120 subsequent binding of the mAB F101.69 is inhibited (Fig. 1A).

Saturation curves of mAB F101.69 on PBMC (4 experiments with different donors) or CEM cells indicated the minimum saturation concentration at 0.3 µg/ml. Therefore a concentration of 10 µg/ml, which was used for CD4 blockade, was more than 1 log above the minimal saturation concentration in FACS analysis (Fig. 1B).

. Gp120 binding on CD4+ CEM cells in presence of blocking mAB F101.69 or of 10% FCS or both : the results are illustrated on figures 2A and 2B.

Gp120 binding to CEM cells was studied at three concentrations 0.5, 1 and 2 µg/ml.

A) (i) CEM cells were incubated with 10 µg/ml anti-CD4 mAB F101.69 (30 min. at 37°C), a concentration more than 1 log over saturation concentrations, in order to block all CD4 receptors and then further incubated with rgp120 (black squares). (ii) Controls were in BSA 3% only (open squares). (iii) rgp120 was pre-incubated with 10% FCS (for 1 h at 37°C) and further incubated with the cells in presence of 10% FCS (open triangles) or (iv) both treatments, on the cells and on rgp120, were effected simultaneously : cells were incubated with blocking mAB F101.69 and gp120 was pre-incubated with FCS 10% (black triangles). rgp120 was detected with a rabbit anti-gp120 antiserum and revealed with an anti-rabbit Ig-phycoerythrin antiserum for analysis in a cell sorter. (mean values of 6 independent experiments, as compared by the intern reference of 2 µg/ml rgp120).

B) The same results are represented as percent rgp120 binding or percent inhibition, as compared to the controls for each rgp120 concentration in BSA 3%.

With increasing gp120 concentrations binding increased about 3 fold (Fig. 2A). The preincubation of gp120 with 10% FCS diminished its binding to cells to about 60% (Fig. 2B). In the presence of FCS, the CD4 receptors were blocked by mAB F101.69 and gp120 binding was reduced to 5%, indicating binding essentially to CD4 (Fig. 2B).

In experiments with BSA 3% the blockade of the CD4 receptor by mAB F101.69 reduced gp120 binding to about 20% at all three concentrations, (Fig. 2B) indicating that 20% of total gp120 bound to secondary binding sites, different from the known CD4 binding domain (6 independent experiments). The percentage found by FACS is inferior to the one by ELISA, but confirms the existence of secondary binding sites by an independent method.

. Binding of rgp120 to CEM cells after their treatment under different conditions (FACS)

- CD4 deletion followed up by FACS

In order to further demonstrate the existence of two different cellular receptors for gp120, experiments were carried out to destroy one of them by enzymatic or chemical methods without affecting the other. CEM cells were exposed to trypsin or paraformaldehyde at different concentrations and then analyzed by FACS for the presence of the CD4 receptor and for continuing gp120 binding. Dead cells, labeled by PI uptake, were excluded. CD4 and gp120 were stained with different fluorochromes, the anti-CD4 mouse mAB F101.69 was revealed with an anti-mouse IgG and the anti-gp120

rabbit antiserum by an anti-rabbit Ig-PE (phycoerythrine).

A) Living CEM cells were treated with trypsin and, after washing, resaturated with BSA 3 %. Treated cells were incubated with the anti-CD4 mAB F101.69 revealed by an anti-mouse Ig FITC for analysis in a FACs.

The results are given on figure 3A to visualize total CD4 (open triangles) or treated cells were incubated with rgp120 (HIV-1 IIIB) from baculovirus and further revealed with rabbit anti-gp120 serum, followed by anti-Ig phycoerythrin (PE) to show gp120 binding (open squares). Finally trypsin treated cells were blocked at the CD4 with mAB F101.09 (at 1 log over saturation concentration) before incubation with gp120, which was then revealed with rabbit anti-gp antiserum and anti-rabbit Ig-PE, as above (black squares)

B) and C) The target cells were treated with paraformaldehyde (PFA) up to 10 mg/ml for 30 min. at 37°C, in order to get pore-formation and access to intracellular binding sites. These experiments were performed in BSA only. After resaturation, the cells were blocked by mAB F101.69 at the CD4 receptor and then double labeled for propidium iodide uptake and for the presence of gp120.

The results are illustrated on figures 3B and 3C.

- CEM cells were treated with paraformaldehyde (PFA) at different concentrations for 30 min. at 37°C. After resaturation with BSA 3%, the CD4 of target cells was blocked with mAB F101.69 (30 min. at 37°C) and thereafter incubated with gp120 for 30 min. at 37°C. Gp120 was detected with an anti gp120 rabbit antiserum (30 min. at 4°C) followed by an anti-rabbit Ig-FITC (30

min. at 4°C). Propidium iodine uptake was simultaneously revealed before analysis in a cell sorter. (means values of 3 independent experiments, compared by the intern reference of 2 µg/ml rgp120).

- Dead cells contained in the experiment or dried cells or PFA treated dead cells showing PI uptake were blocked or no blocked with mAB F101.69, and further incubated with rgp120 as in Fig.3B for FACS analysis.

At low PFA concentrations, up to 0.1 mg/ml, PI uptake was low and increased rapidly between 1 and 10 mg/ml PFA. Binding of gp120 increased in a similar way between PFA concentrations of 1 - 10 mg/ml (Fig. 3B). Since CD4 was blocked by mAB F101.69, only gp120 bound to secondary receptors was measured. Therefore secondary receptors are more abundant in permeabilized cells, probably intracellularly, or they possess higher affinities.

Specific gp120 binding to secondary sites was found to be increased in different kinds of permeabilized cells. In dried cells, PFA treated cells, or permeabilized cells, occurring during the experiment, 60%-70% of total bound gp120 were fixed to secondary binding sites by FACS analysis (Fig. 3C).

- CD4 deletion followed up by ELISA

Deletion of CD4 was studied in parallel by the ELISA method, on microtiter plate adsorbed dried cells, which allow more drastic treatment of cells with detergents. It was first verified with specific mABs, that a variety of CD antigens resisted to the drying procedure of CEM cells (18h, 37°C). CD4, CD5 and CD7 were preserved after drying, so that dried CEM cells presenting intact CD4 binding sites, could be treated under the same conditions as living cells with enzymes,

PFA and even detergents to destroy CD4 receptor integrity.

. treatment with trypsin (figure 4A)

Trypsin was used at different concentrations, from 0.03 to 0.5 mg/ml.

Microtiter adherent, dried CEM cells were saturated with BSA 3 % and treated with trypsin and after washing and resaturation with BSA 3 % incubated with the anti-CD4 mAB F101.69 linked to peroxidase and then revealed with OPD to show presence of CD4 (open triangles), or dried cells were incubated with rgp120 and revealed with a rabbit anti-gp120 antiserum, followed by anti-rabbit Ig linked to peroxidase and OPD to demonstrate gp120 binding (black squares).

With increasing trypsin concentrations, CD4 detection diminished progressively and disappeared almost completely at 0.25 mg/ml trypsin, very similar to FACS analysis.

The binding capacity of gp120 in the absence of serum to trypsin treated cells did not diminish in ELISA, even after exposure to the highest trypsin concentration (4 experiments). This confirms a higher resistance of the receptors of the invention than CD4 to enzymatic degradation, as already found by FACS nalysis.

. treatment with paraformaldehyde (figure 4B wherein Pi means propidium iodide incorporation).

When dried CEM cells were chemically treated with paraformaldehyde from 0.01 to 100 mg/ml, the integrity of CD4 domain 1 was reduced to only 25 % as revealed with mAB F101.69 Nevertheless, gp120 binding without serum increased slightly (3 experiments). Dried cells in ELISA simultaneously expose extra and intra cellular sites. Thus the increased gp120 binding to the receptors of the invention, found with living CEM cells

FIGURE 4A

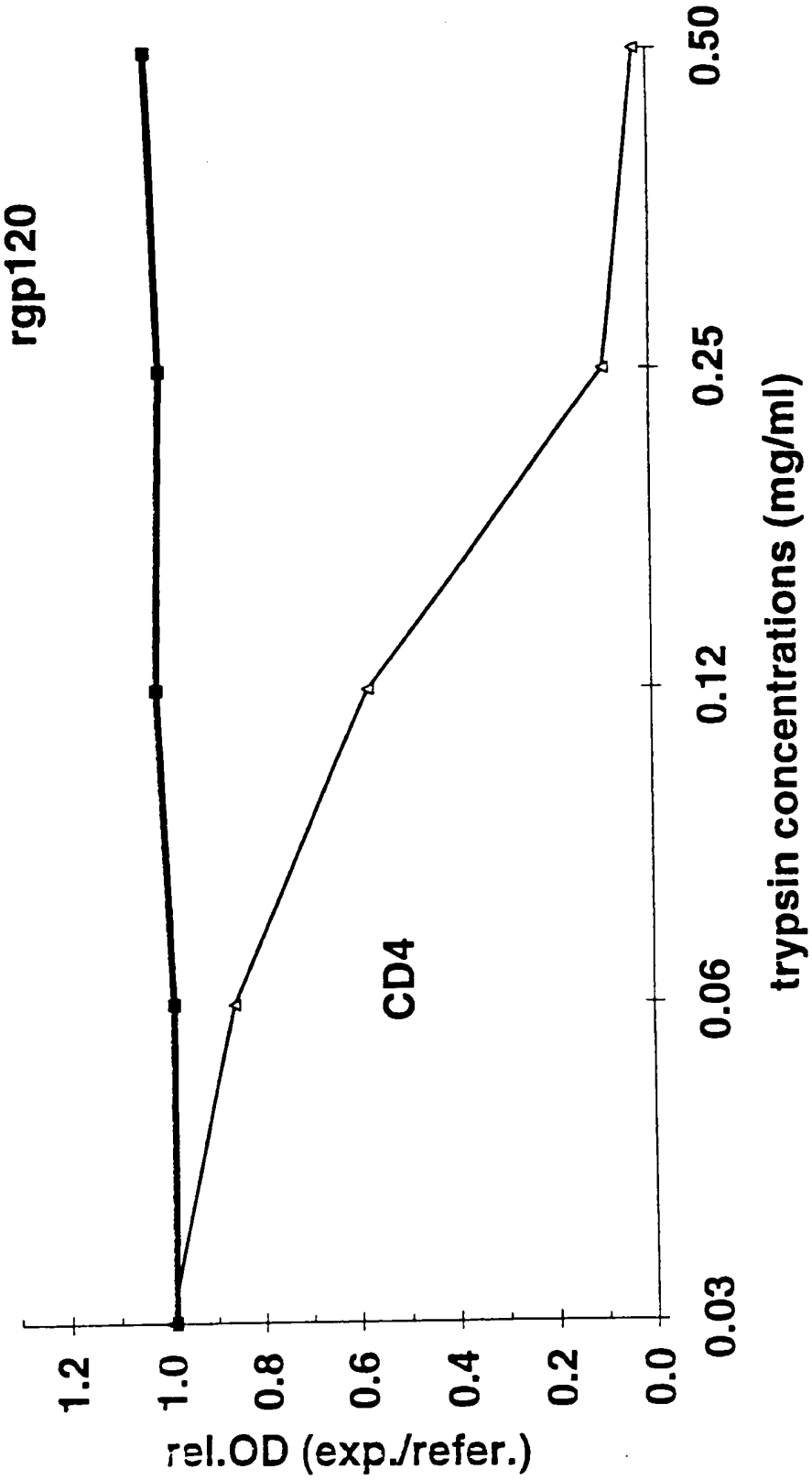

rgp120
CD4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
rel.OD (exp./refer.)
0.03
0.06
0.12
0.25
0.50
trypsin concentrations (mg/ml)

FIGURE 4B

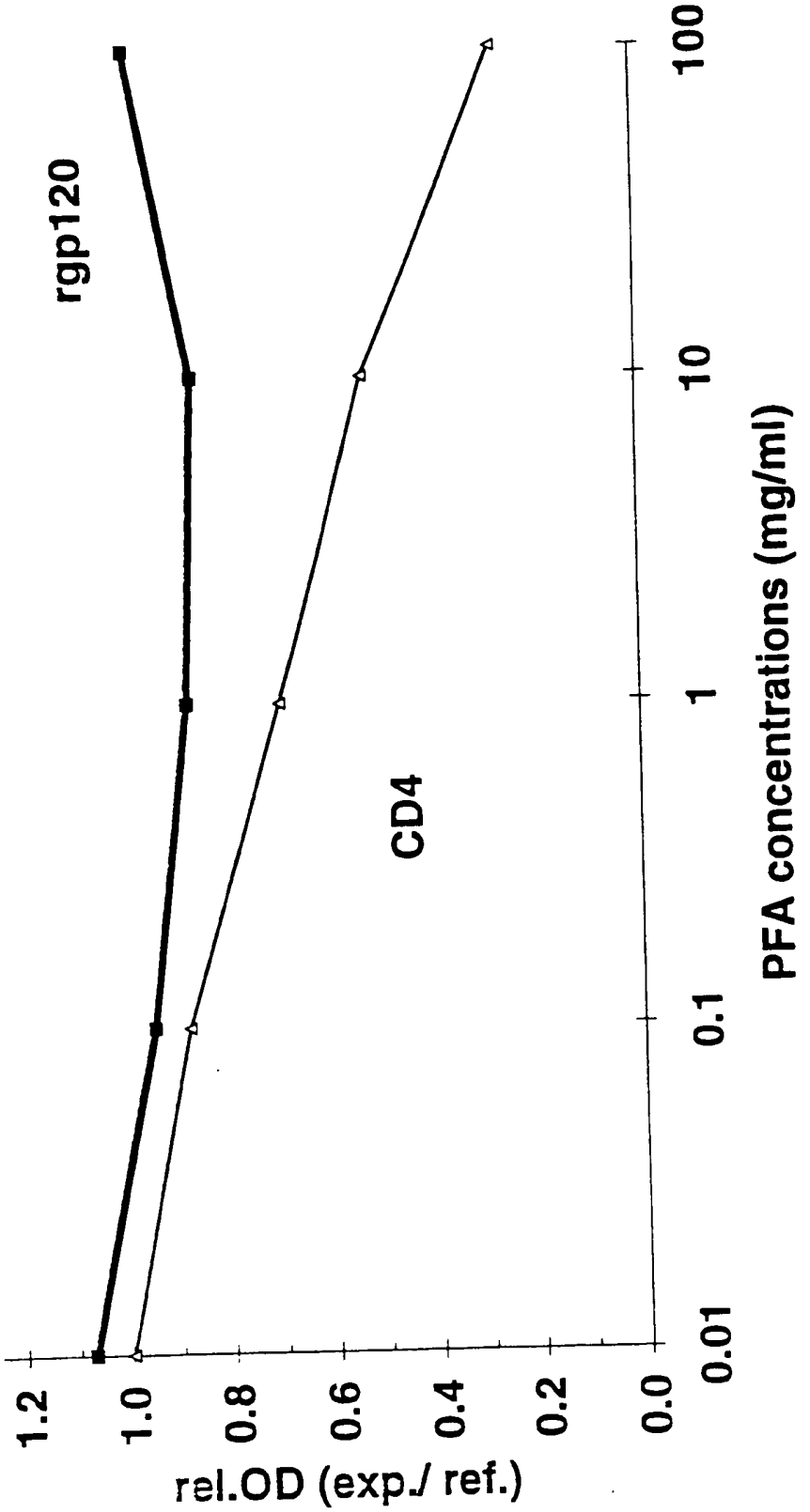

rgp120
CD4
PFA concentrations (mg/ml)
100
10
1
0.1
0.01
1.2
1.0
0.8
0.6
0.4
0.2
0.0
rel.OD (exp./ ref.)

FIGURE 4C

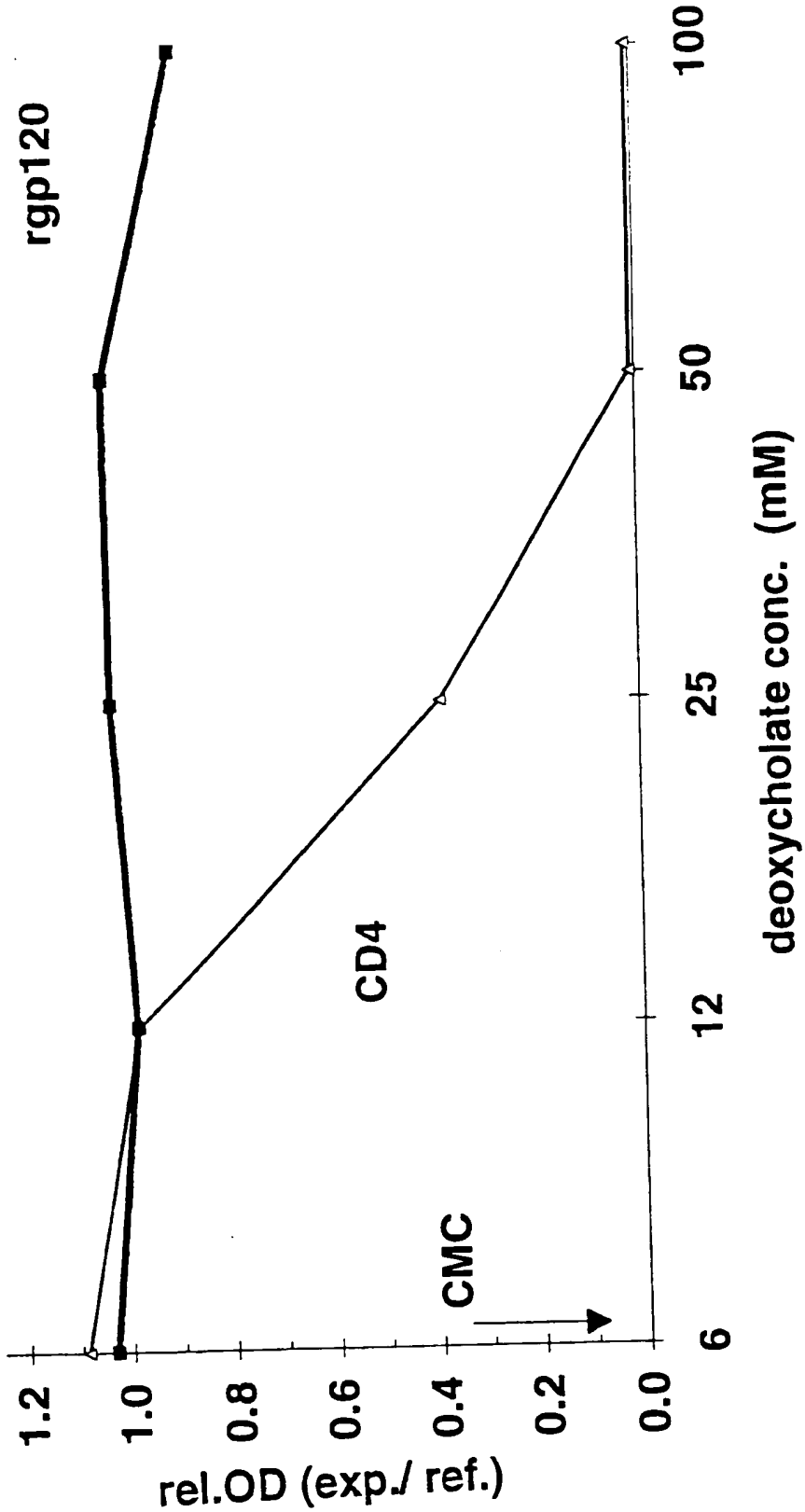

rgp120
CD4
CMC
rel.OD (exp./ ref.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
6
12
25
50
100
deoxycholate conc. (mM)

FIGURE 5A

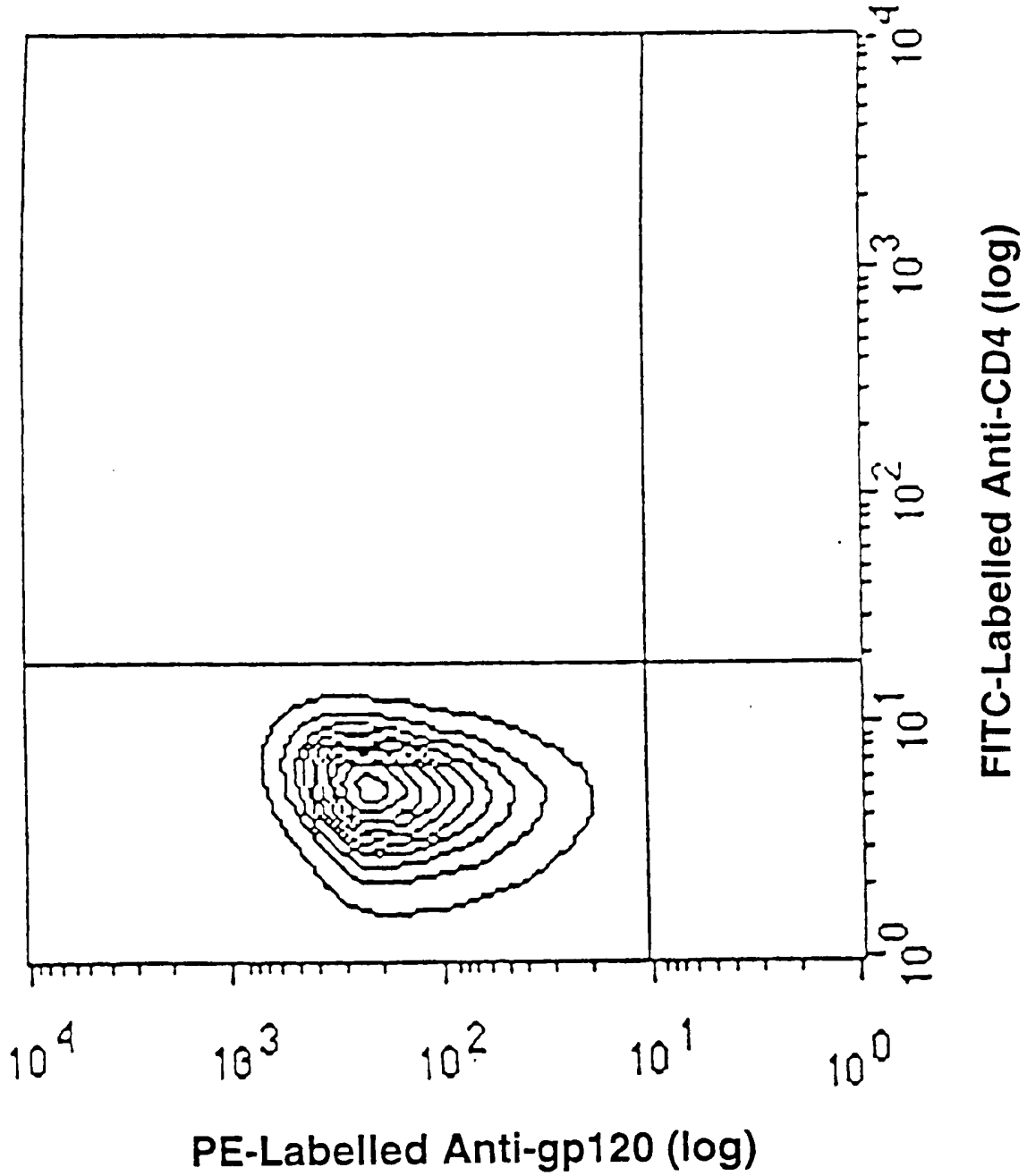

FITC-Labelled Anti-CD4 (log)
PE-Labelled Anti-gp120 (log)
10^0
10^1
10^2
10^3
10^4

FIGURE 5B

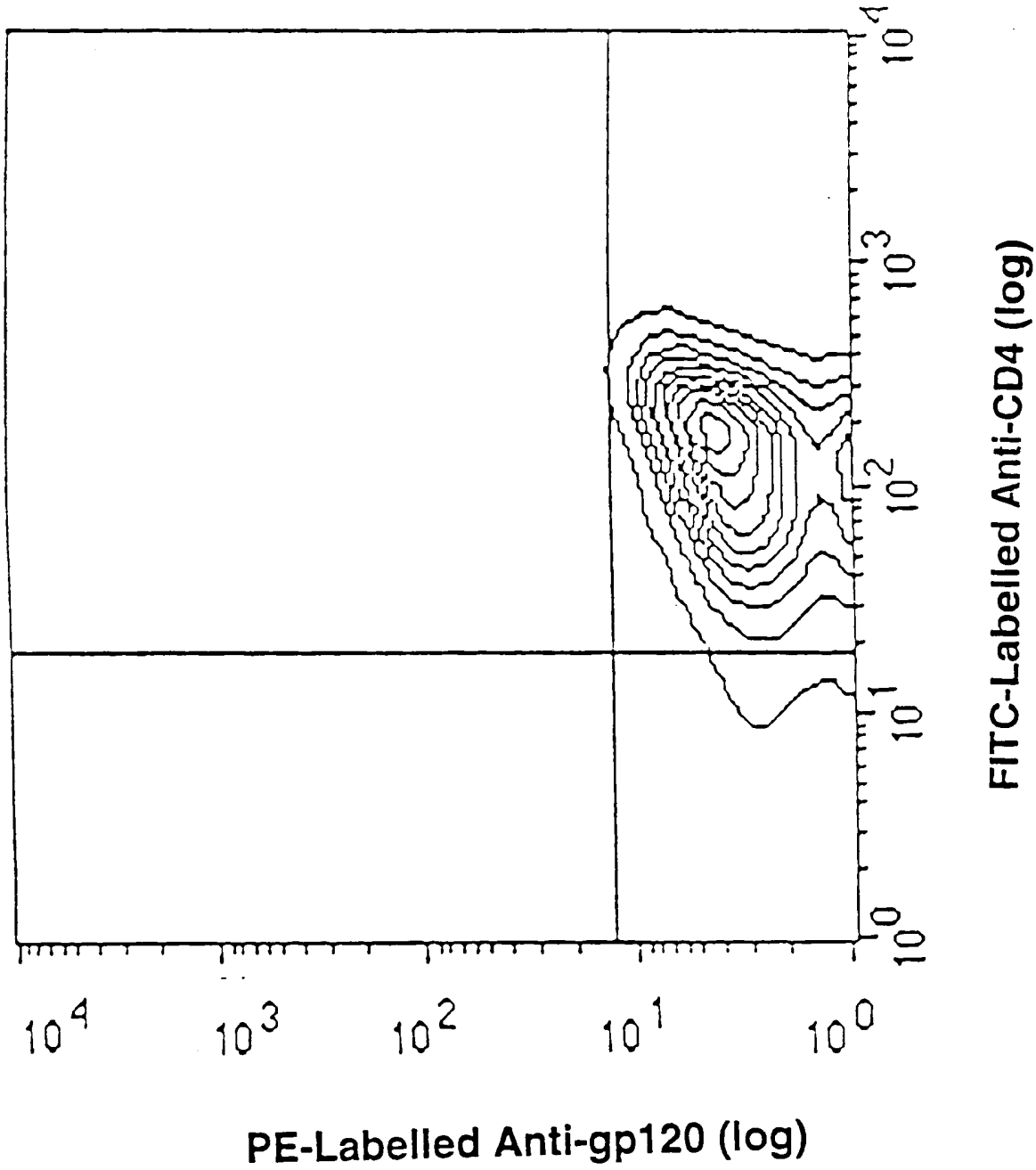

FITC-Labelled Anti-CD4 (log)
PE-Labelled Anti-gp120 (log)
10^0
10^1
10^2
10^3
10^4

FIGURE 5C

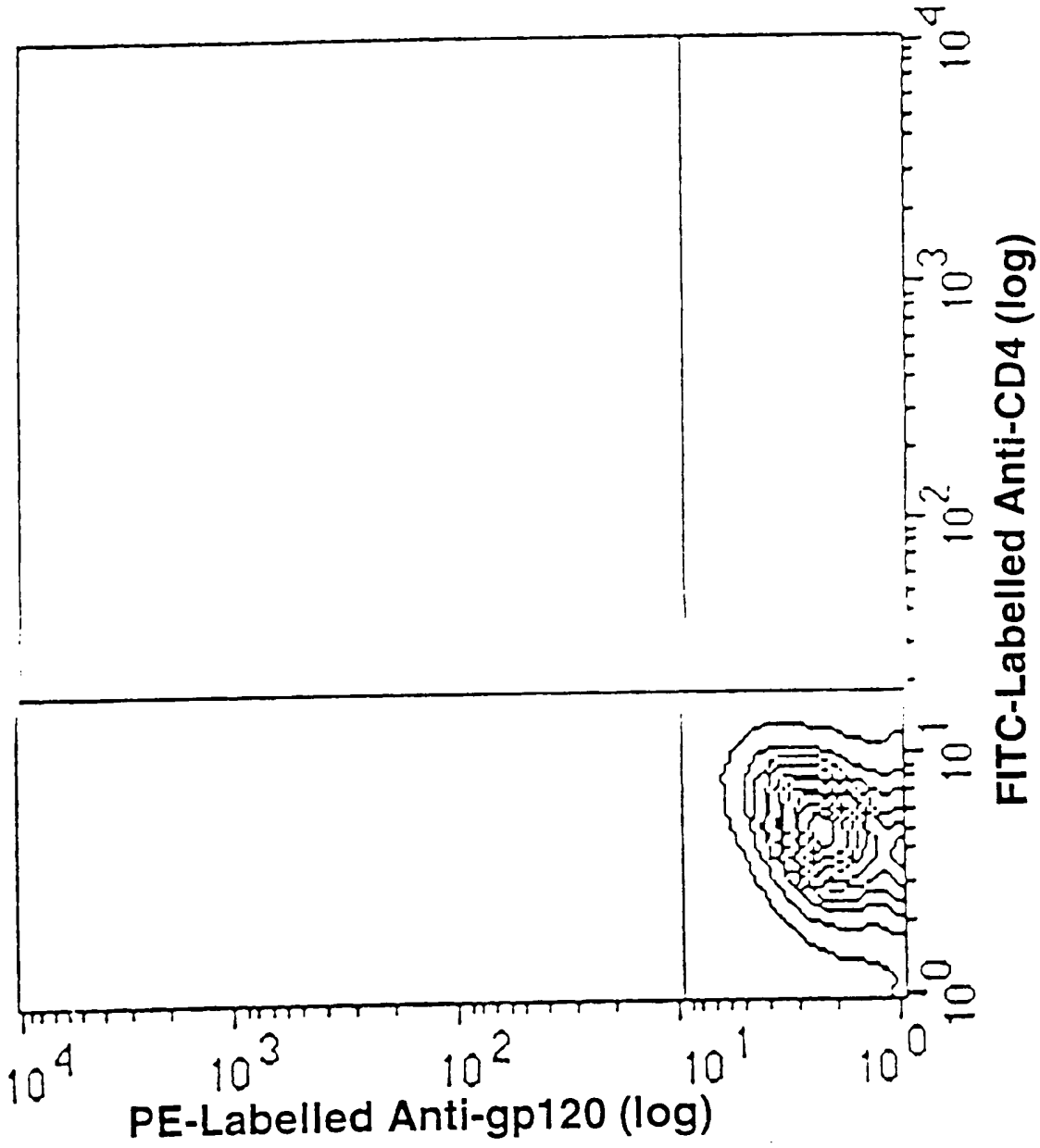
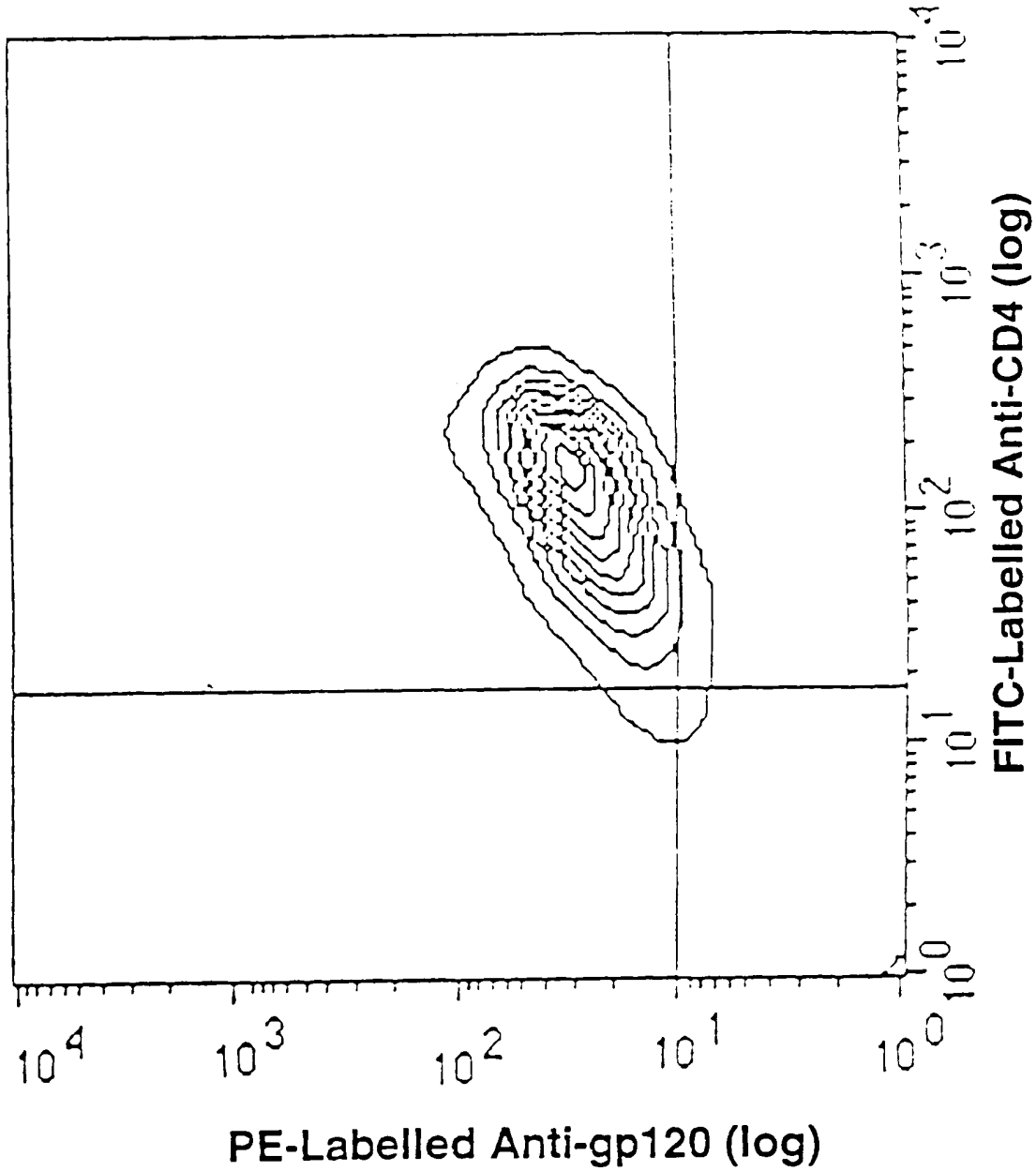

FITC-Labelled Anti-CD4 (log)
PE-Labelled Anti-gp120 (log)
10^0
10^1
10^2
10^3
10^4

FIGURE 5D


FITC-Labelled Anti-CD4 (log)
PE-Labelled Anti-gp120 (log)
10^4
10^3
10^2
10^1
10^0

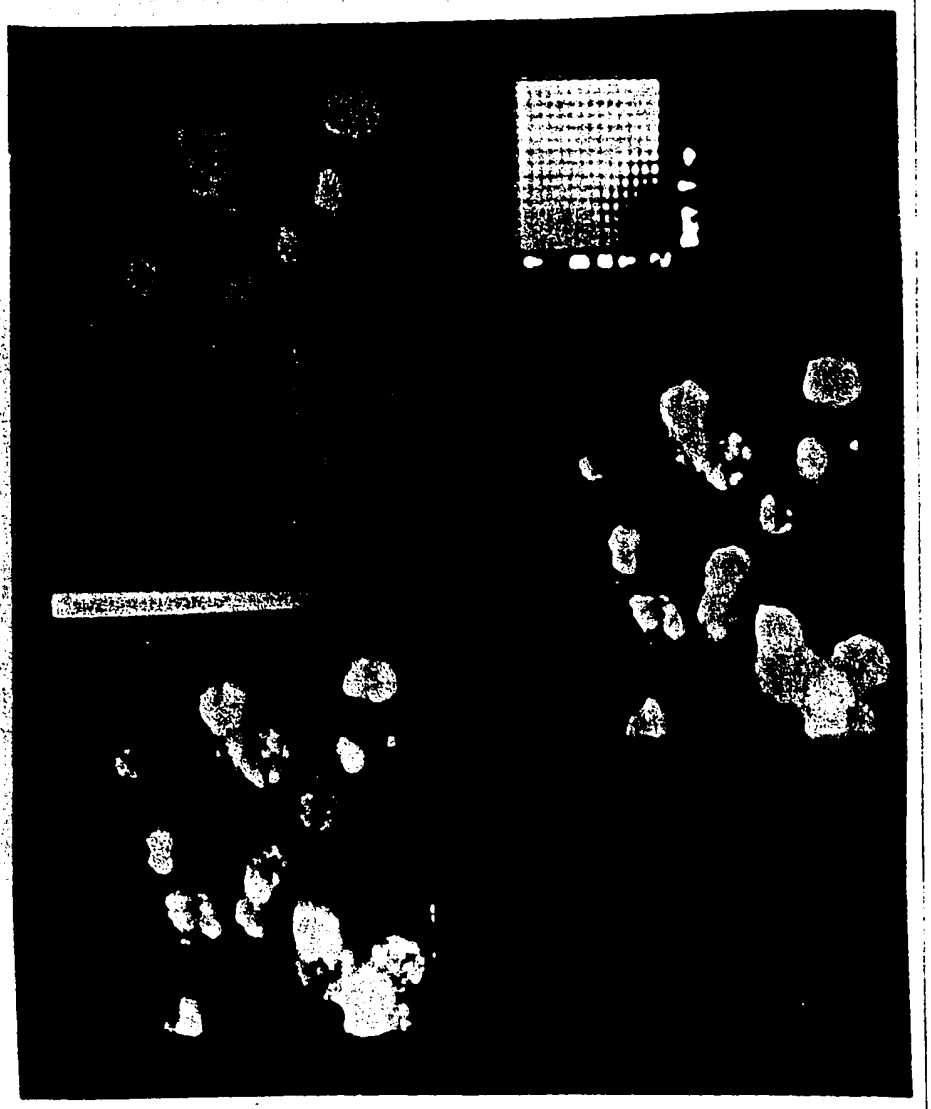


FIGURE 6

FIGURE 7

mAB inhibition of gp120 binding to II. Binding Site

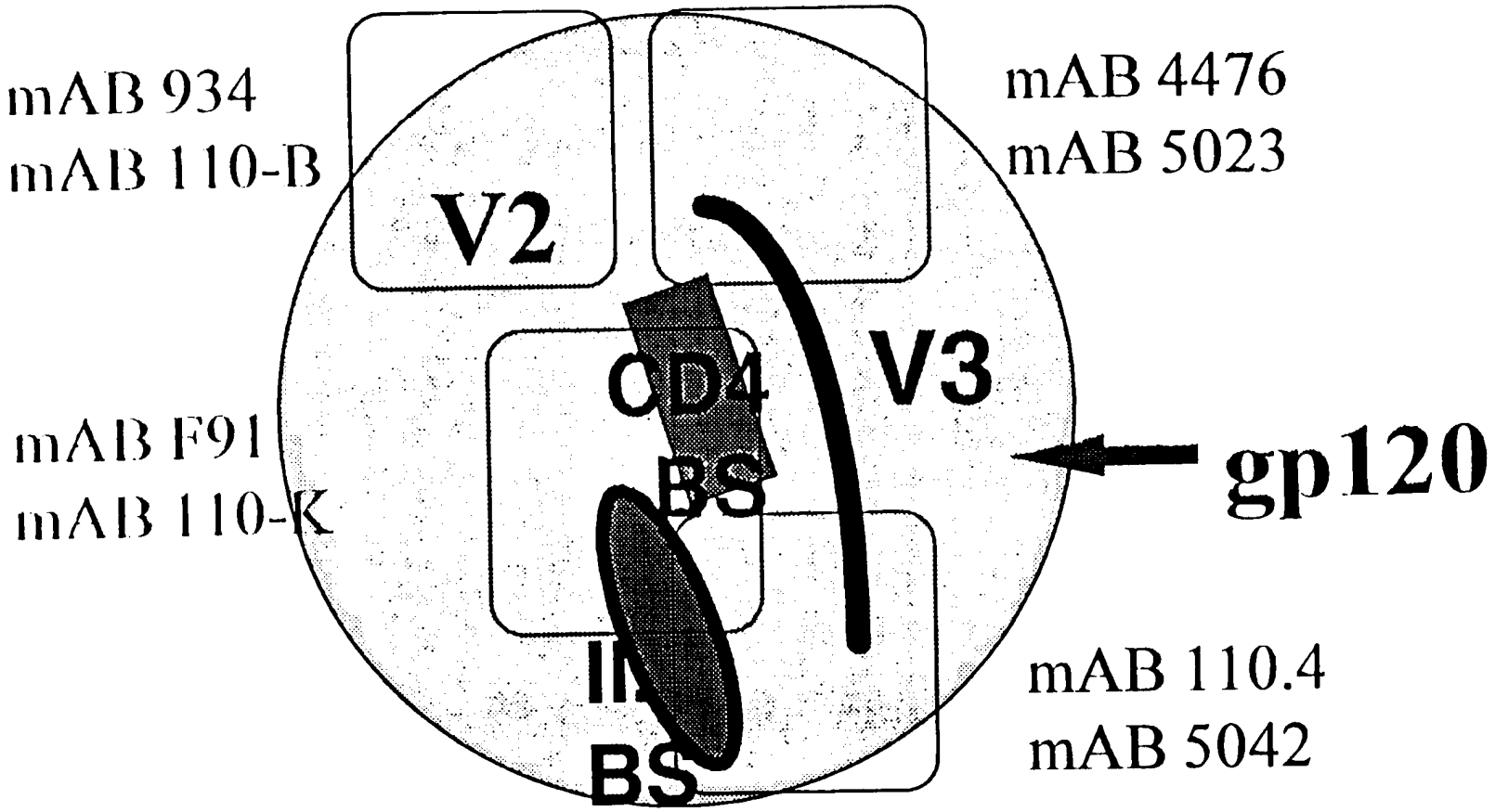


FIGURE 8A

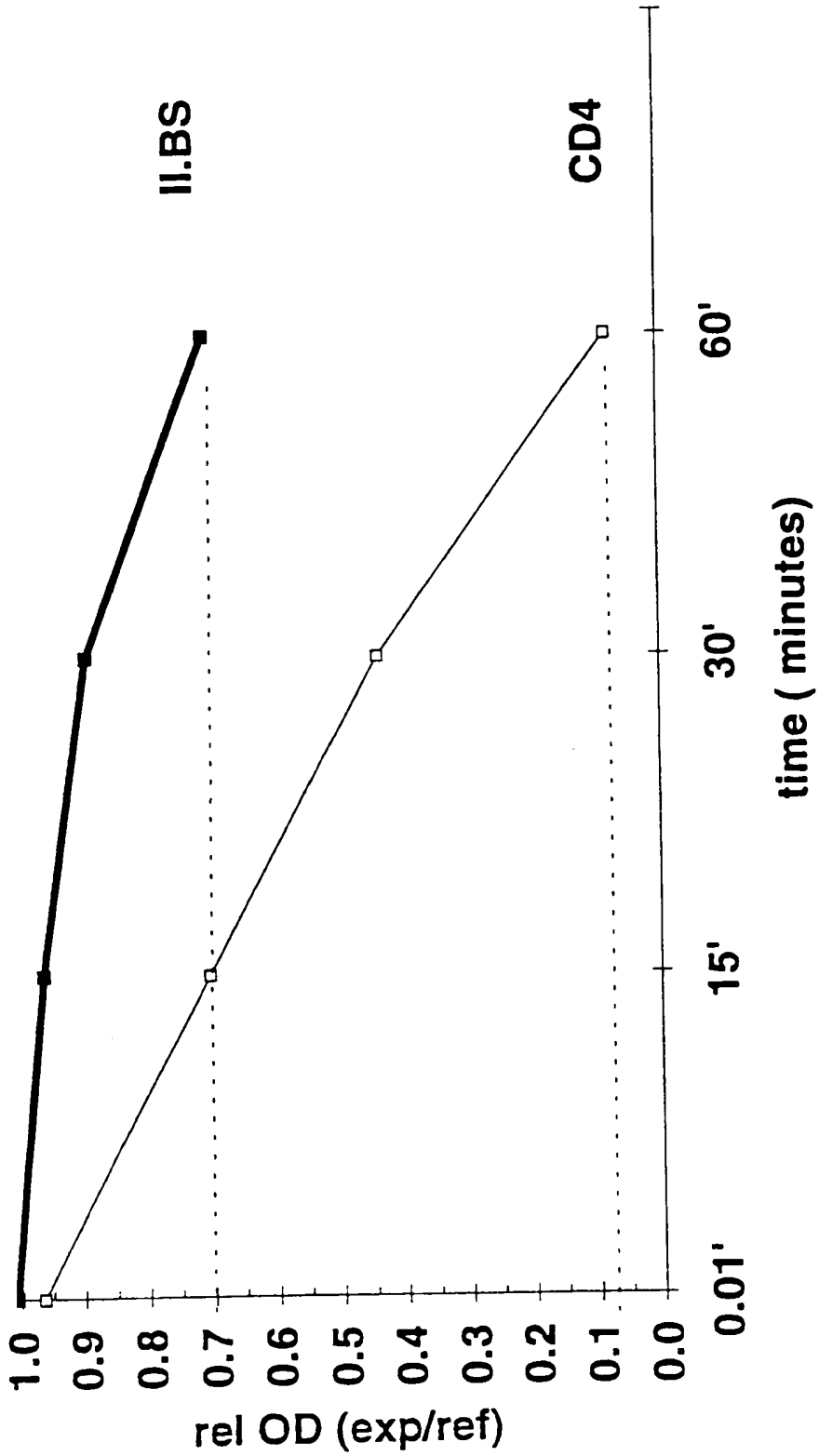

II.BS
CD4
60'
30'
15'
0.01'
time (minutes)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
rel OD (exp/ref)

FIGURE 8B

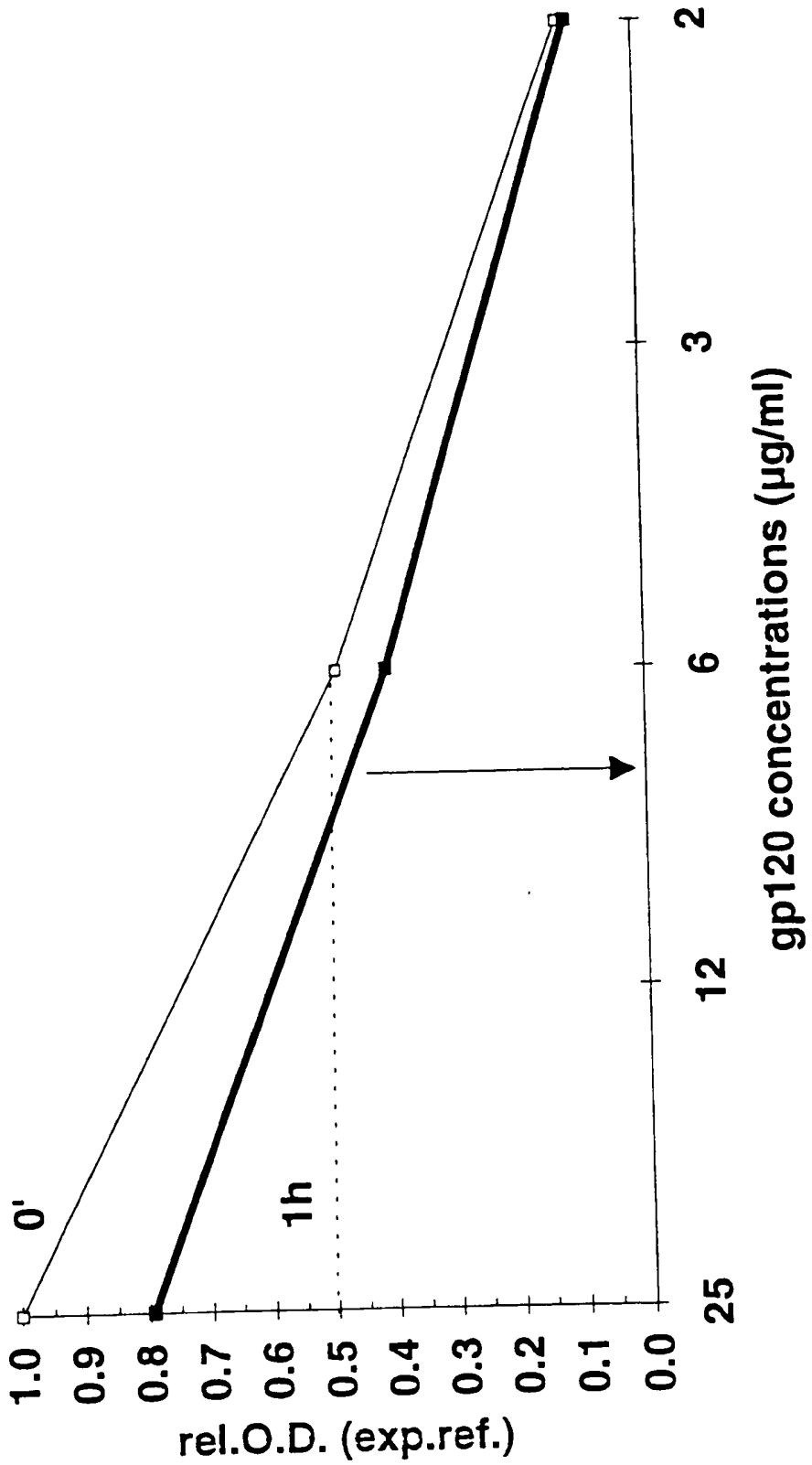
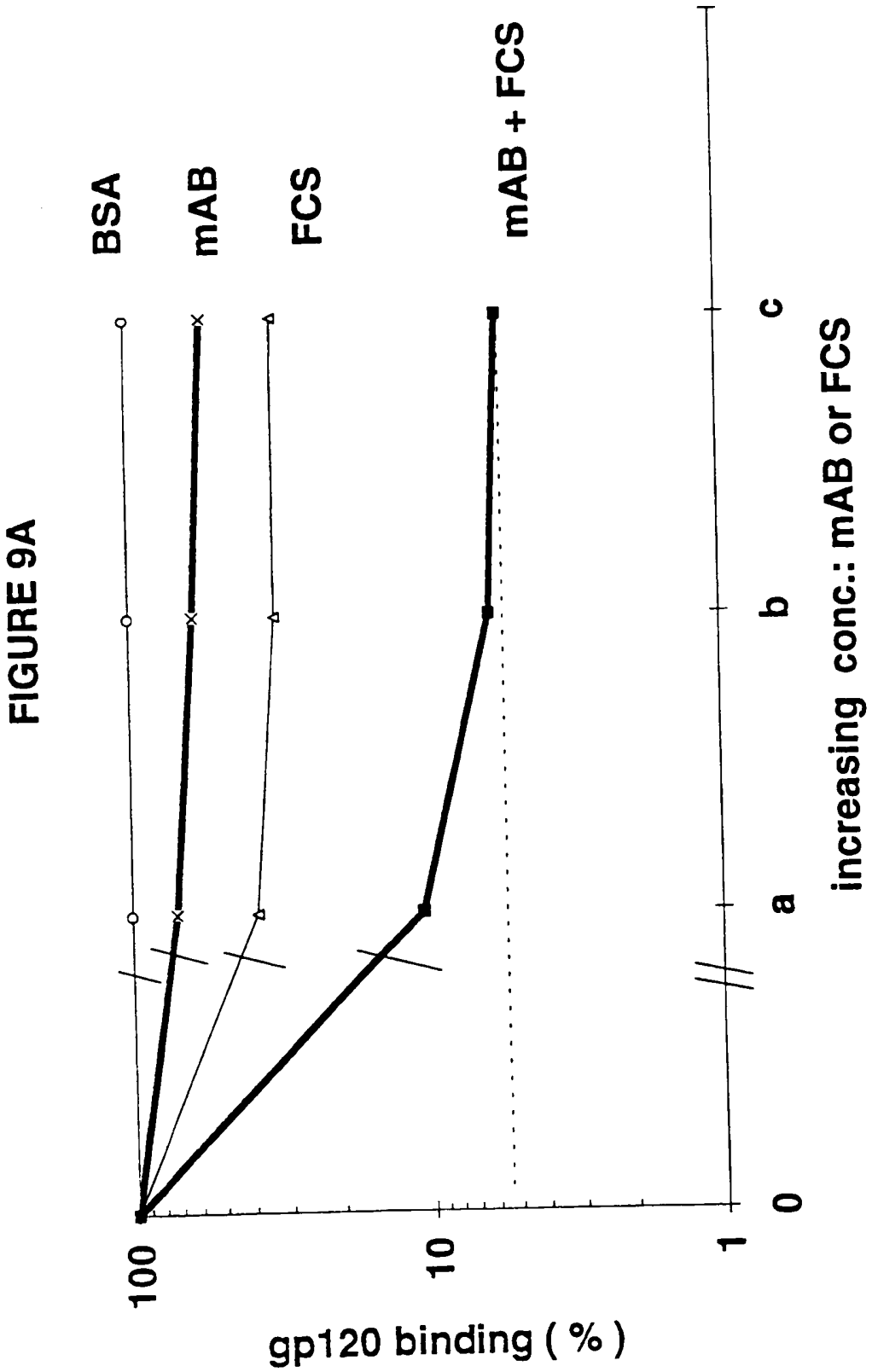

0'
1h
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
rel.O.D. (exp.ref.)
2
3
6
12
25
gp120 concentrations (µg/ml)

FIGURE 9A


BSA
mAB
FCS
mAB + FCS
100
10
1
0
gp120 binding (%)
a
b
c
increasing conc.: mAB or FCS

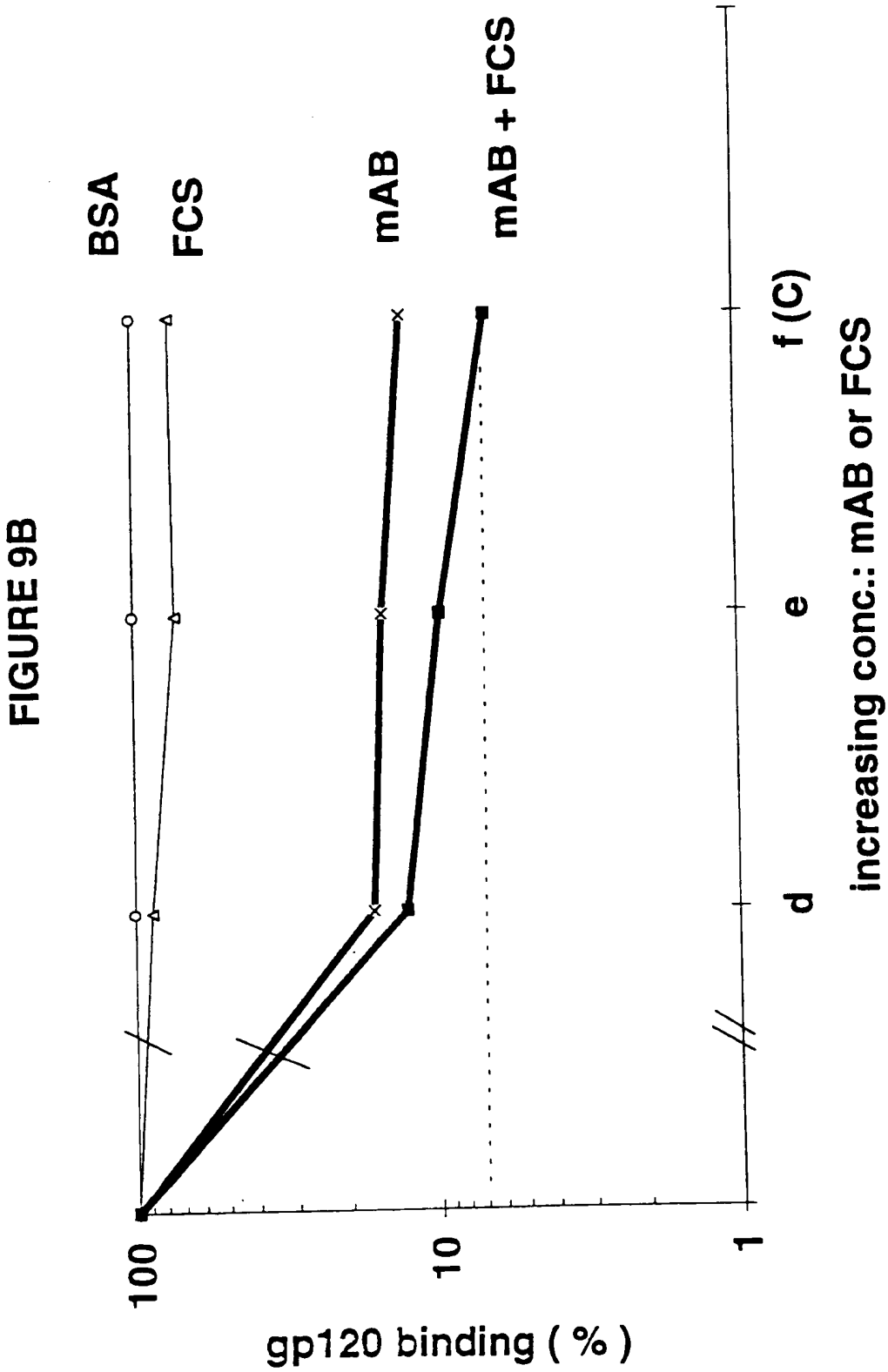

BSA
FCS
mAB
mAB + FCS
100
10
1
gp120 binding (%)
d
e
f (C)
increasing conc.: mAB or FCS

FIGURE 9B

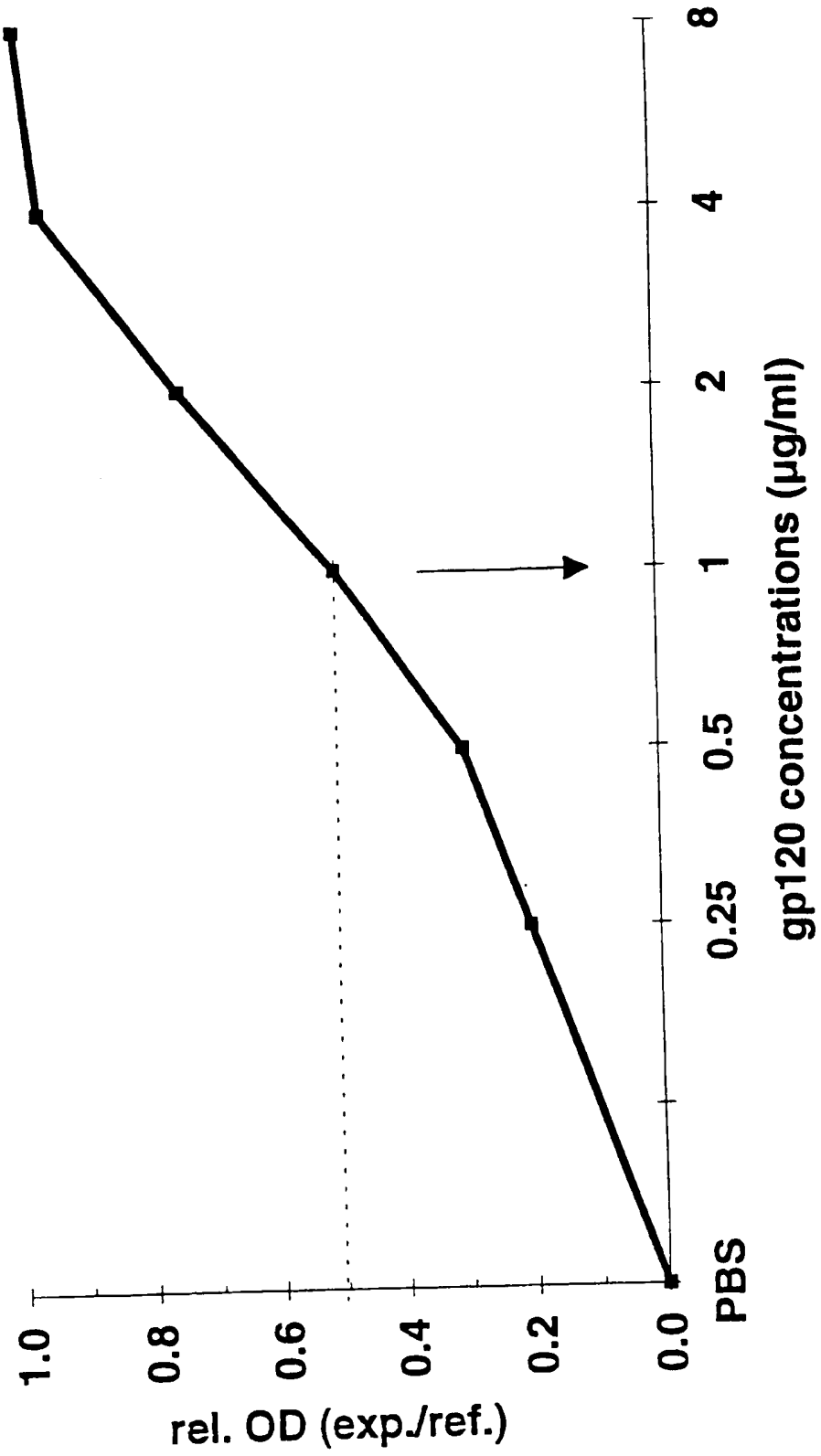

rel. OD (exp./ref.)
1.0
0.8
0.6
0.4
0.2
0.0
PBS
0.25
0.5
1
2
4
8
gp120 concentrations (µg/ml)

FIGURE 10A

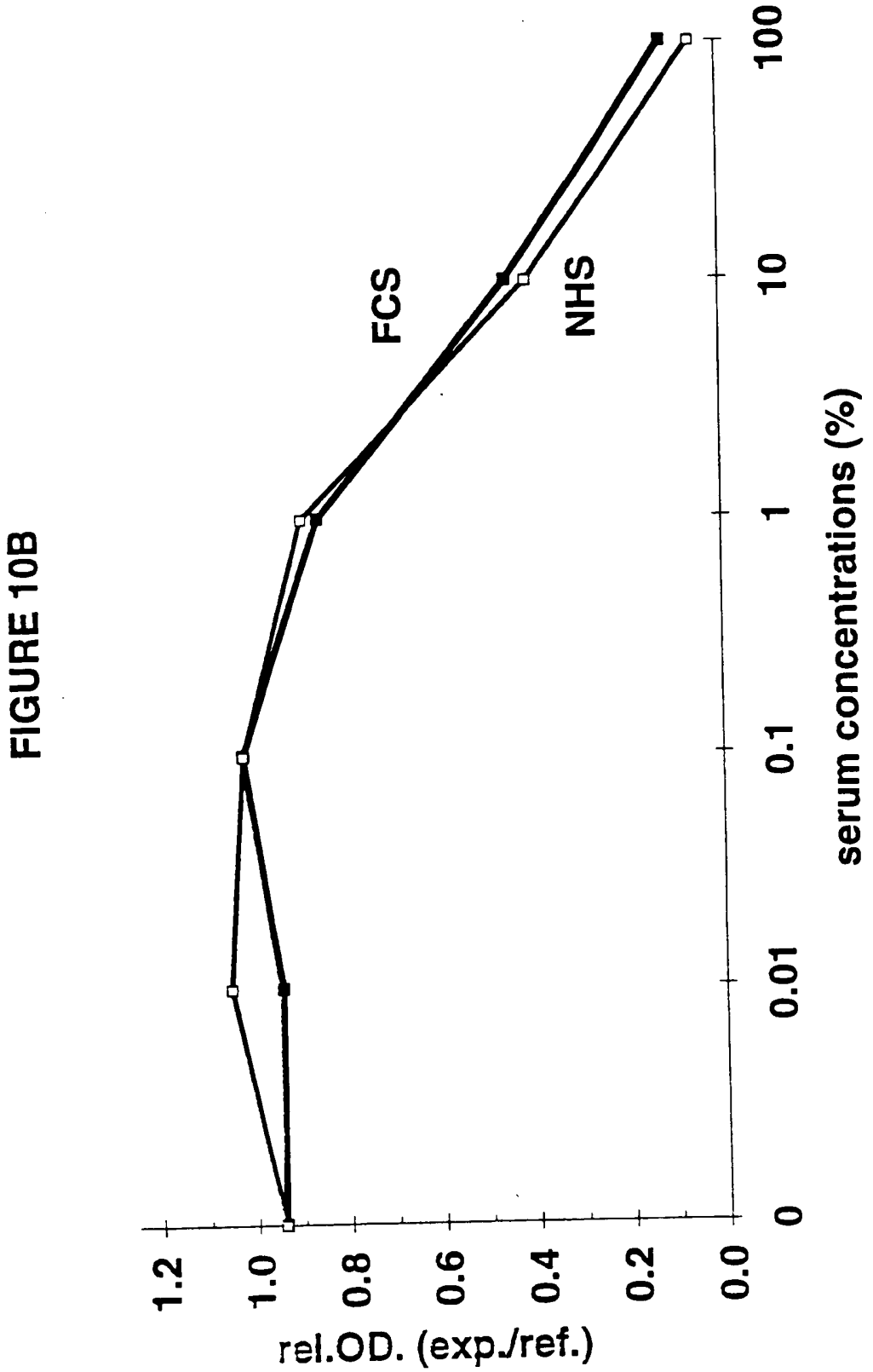

FCS
NHS
100
10
1
0.1
0.01
0
serum concentrations (%)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
rel.OD. (exp./ref.)

FIGURE 10B

FIGURE 11

BAND 3 protein from erythrocytes

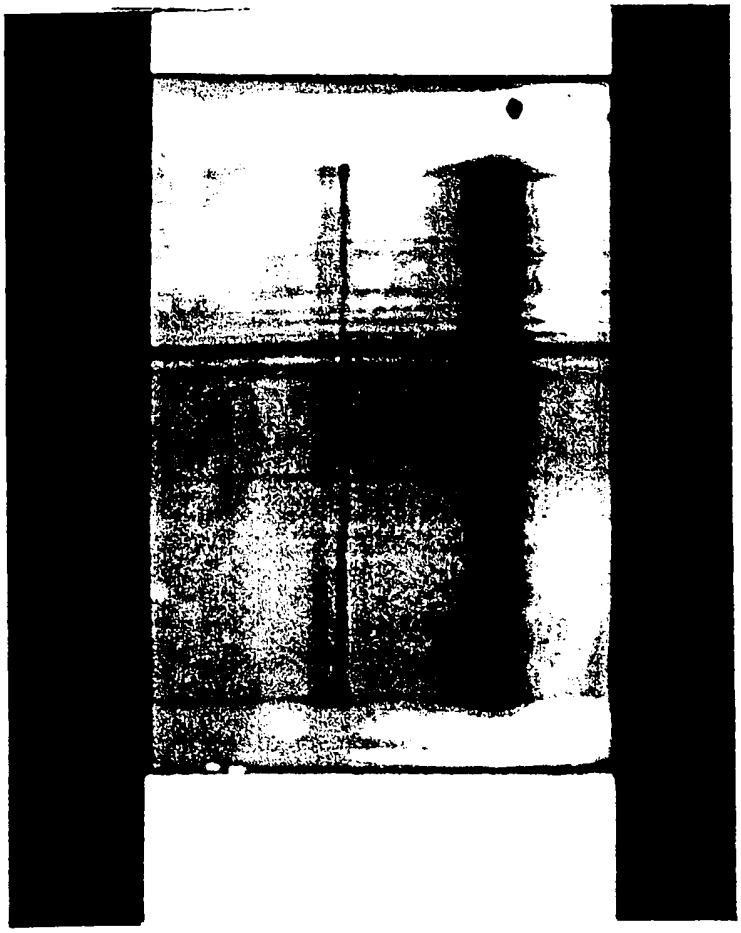


FIGURE 12

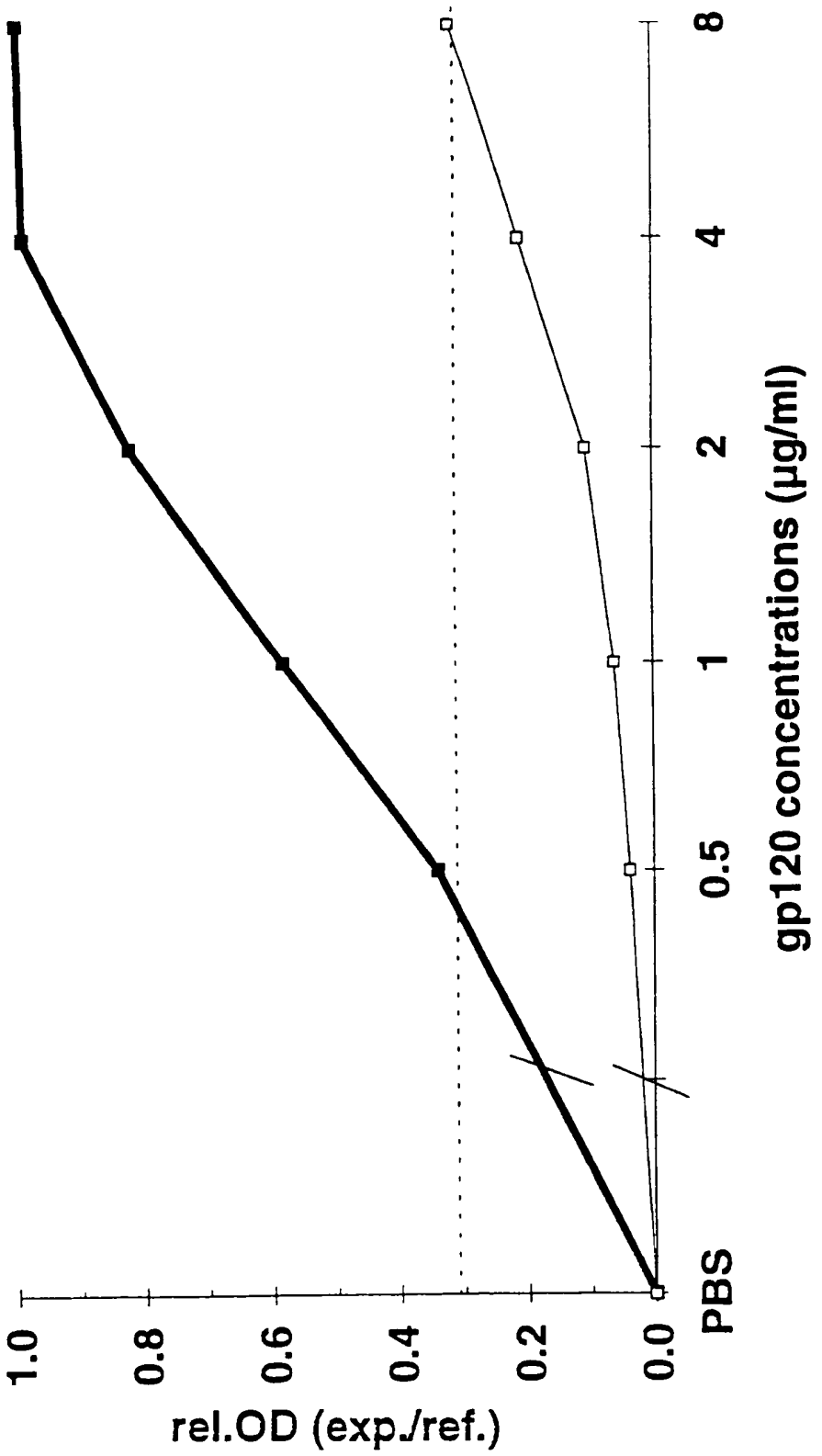
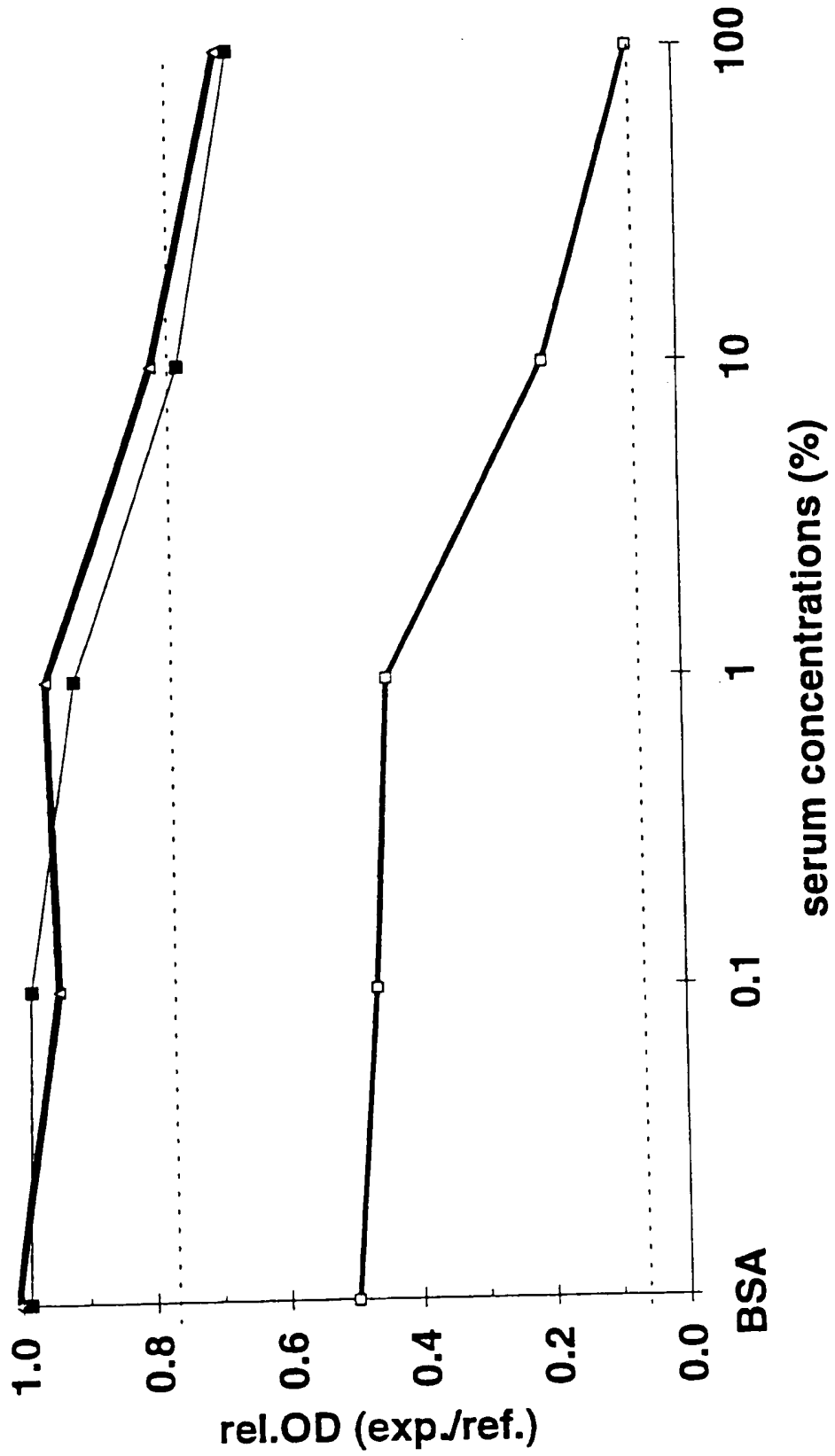

rel.OD (exp./ref.)
1.0
0.8
0.6
0.4
0.2
0.0
PBS
0.5
1
2
4
8
gp120 concentrations (µg/ml)

FIGURE 13A


1.0
0.8
0.6
0.4
0.2
0.0
rel.OD (exp./ref.)
BSA
0.1
1
10
100
serum concentrations (%)

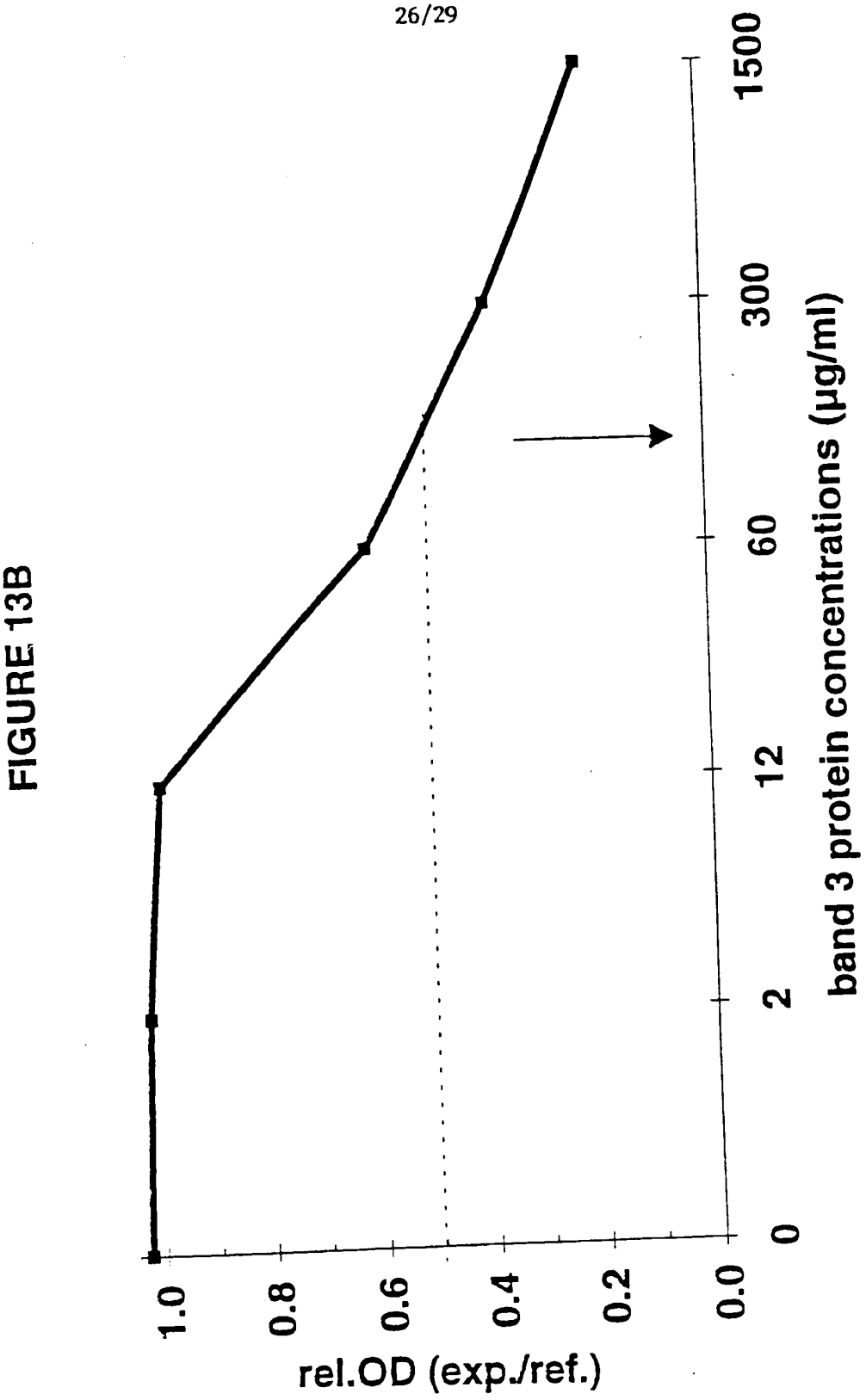

1.0
0.8
0.6
0.4
0.2
0.0
rel.OD (exp./ref.)
0
2
12
60
300
1500
band 3 protein concentrations (µg/ml)

FIGURE 13B

FIGURE 14

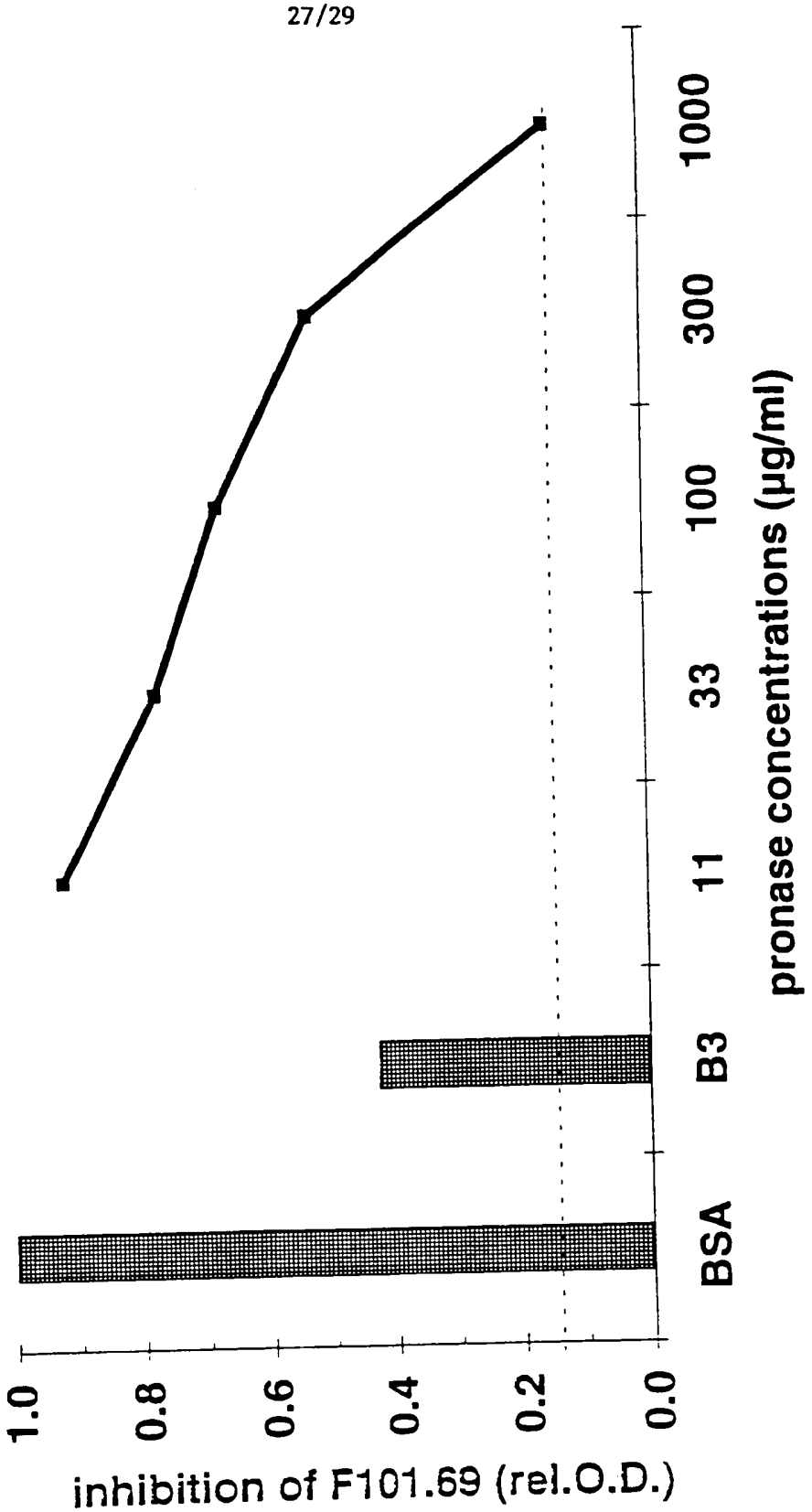

1000
300
100
33
11
pronase concentrations (µg/ml)
B3
BSA
1.0
0.8
0.6
0.4
0.2
0.0
inhibition of F101.69 (rel.O.D.)

FIGURE 15 A

Inhibition of RT after adsorption of HIV to band 3 vesicles

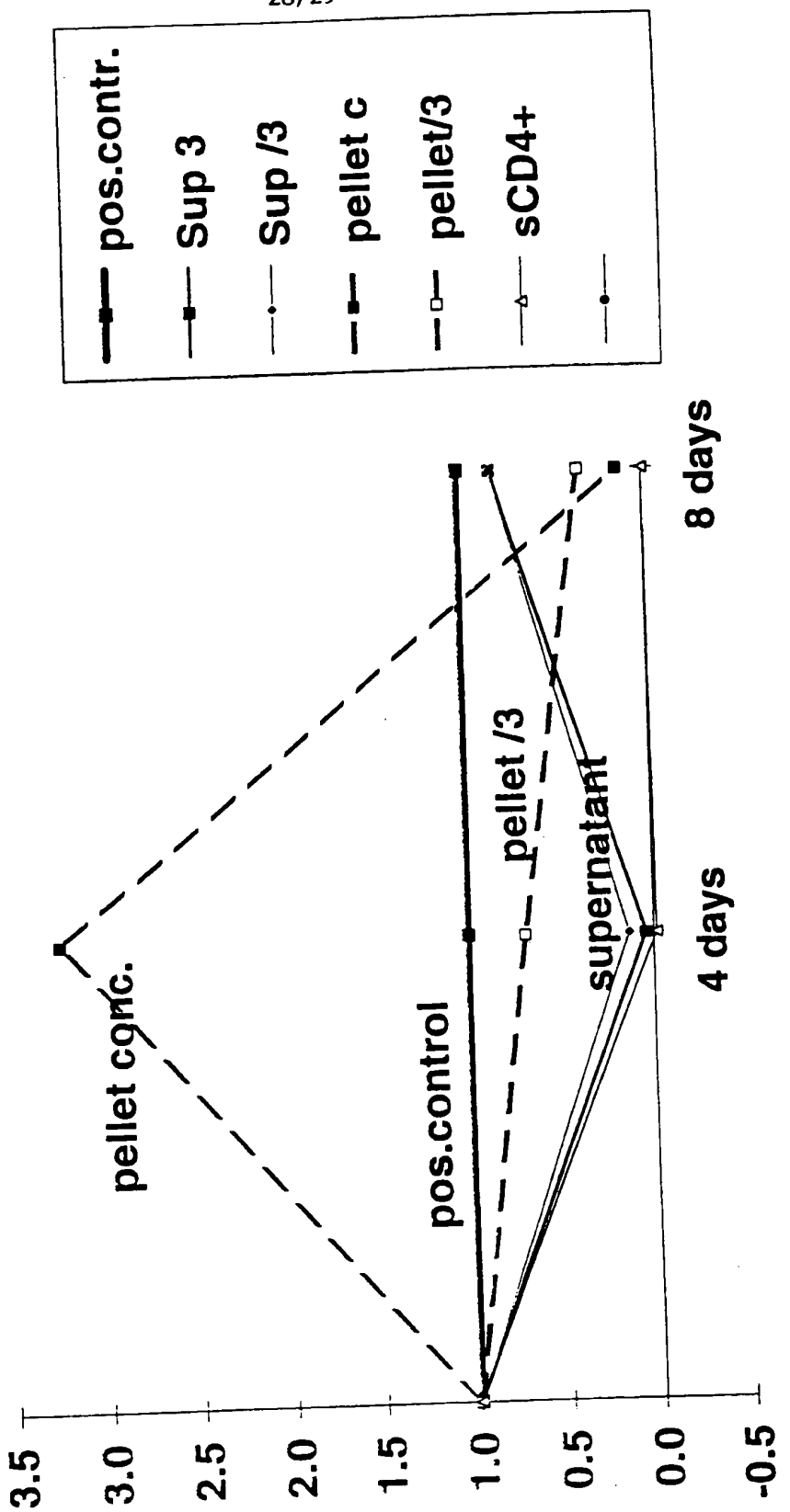
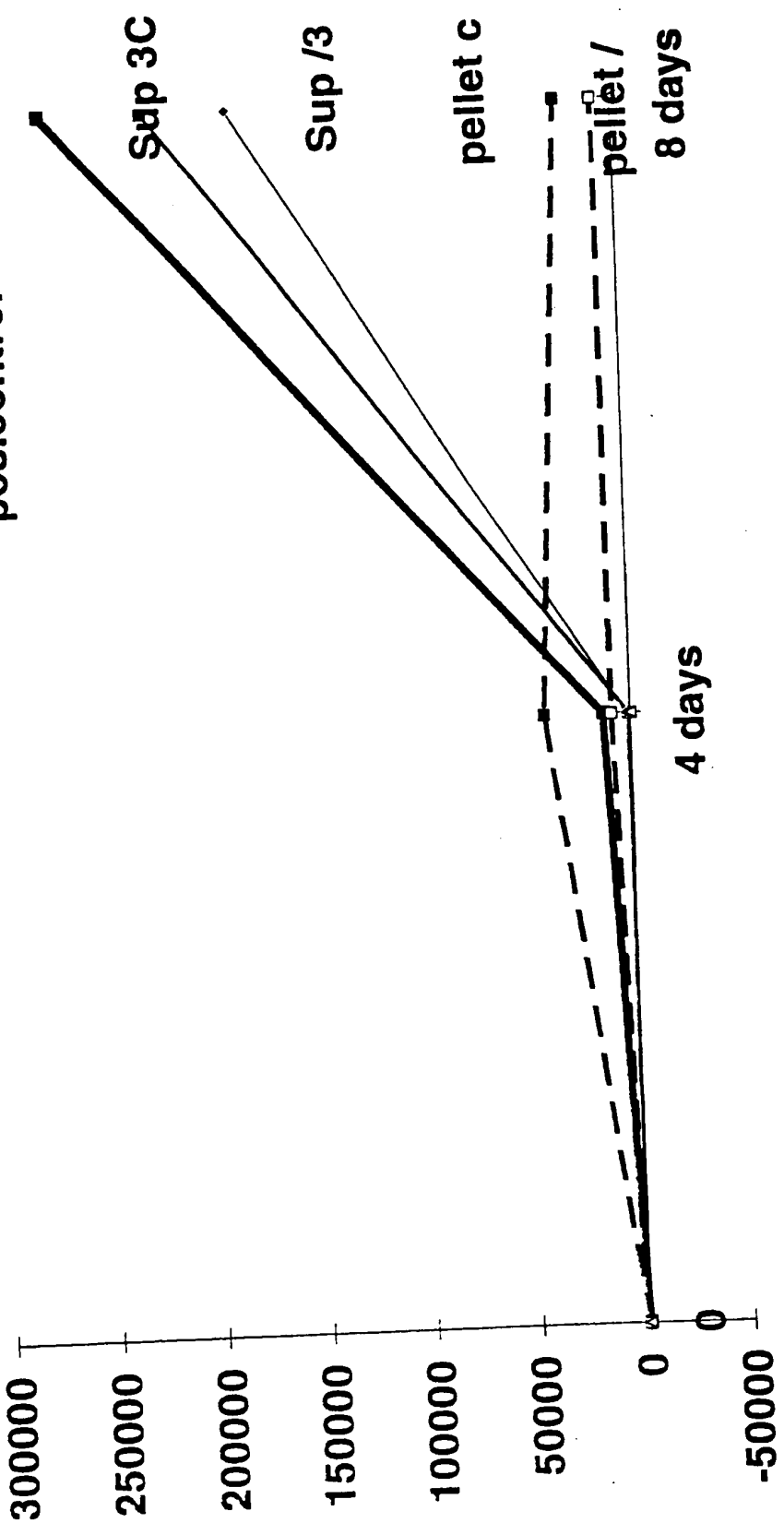


FIGURE 15B

Inhibition of RT after adsorption of HIV to band 3 vesicles



INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/IB 96/00571

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/49 C07K14/73 C07K14/16 C07K14/725 C07K16/08
//A61K35/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,93 01820 (SQUIBB BRISTOL MYERS CO) 4 February 1993 see the whole document ---	1-27
Y	WO,A,94 28915 (PASTEUR INSTITUT ;HOVANESSIAN ARA (FR); CALLEBAUT CHRISTIAN (FR);) 22 December 1994 see the whole document ---	1-27
A	JOURNAL OF VIROLOGY, vol. 67, no. 7, July 1993, pages 3978-3988, XP000579277 M. THALI ET AL.: "Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding." see the whole document ---	22-24

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 September 1996	04. 10. 96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Hix, R

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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	THE JOURNAL OF IMMUNOLOGY, vol. 149, no. 5, 1 September 1992, pages 1779-1787, XP000578391 L.C. BURKLY ET AL.: "Inhibition of HIV infection by a novel CD4 domain 2-specific monoclonal antibody." see the whole document ---	1-27
P,A	VIROLOGY, vol. 211, no. 2, 20 August 1995, pages 583-588, XP000578383 A.L. DEVICO ET AL.: "Monoclonal antibodies raised against covalently crosslinked complexes of human immunodeficiency virus type 1 gp120 and CD4 receptor identify a novel complex-dependent epitope on gp120." see the whole document -----	16,25

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Ir ational Application No
PCT/IB 96/00571

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		PT-A-	100694	29-10-93
		ZA-A-	9205305	14-06-93
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